AD-A060 569 CINCINNATI UNIV OHIO COLL OF MEDICINE F/G 6/13
HOST DEFENSE AGAINST OPPORTUNIST MICROORGANISMS FOLLOWING TRAUM--ETC(U)
JUN 78 A B BJORNSON, W A ALTEMEIER DAMD17-76-C-6023
UNCLASSIFIED NL

1 OF 2
AD
A060569

REPORT #3

"Host Defense Against Opportunist Microorganisms Following Trauma"

ANNUAL SUMMARY REPORT

Ann B. Bjornson, Ph.D.
William A. Altemeier, M.D.
H. Stephen Bjornson, M.D., Ph.D.

JUNE, 1978

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

Fort Detrick,
Frederick, Maryland 21701

Contract No. DAMD-17-76-C-6023

University of Cincinnati

Cincinnati, Ohio 45221

"Approved for public release; distribution unlimited."

"The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents."

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER: Annual Summary Rept. 1 Jul	2. GOVT. ACCESSION NO.
3. RECIPIENT'S CATALOG NUMBER: 77-30 June 78	
4. TITLE (and Subtitle): HOST DEFENSE AGAINST OPPORTUNIST MICROORGANISMS FOLLOWING TRAUMA •	5. TYPE OF REPORT & PERIOD COVERED: Annual Summary Report, July 1, 1977 to June 30, 1978
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s): Ann B. Bjornson, Ph.D. William A. Altemeier, M.D. H. Stephen Bjornson, M.D., Ph.D.	8. CONTRACT OR GRANT NUMBER(s): DAMD-17-76-C-6023
9. PERFORMING ORGANIZATION NAME AND ADDRESS: University of Cincinnati Cincinnati, Ohio 45267 College of Medicine	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: 61102A 35161102 BSO.00.014
11. CONTROLLING OFFICE NAME AND ADDRESS: U.S. Army Medical Research and Development Command Fort Detrick, Frederick, Maryland 21701	12. REPORT DATE: June, 1978 13. NUMBER OF PAGES: 148
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office): 151 p.	15. SECURITY CLASS. (of this report): Unclassified 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

"Approved for public release; distribution unlimited."

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

Portions of this report have been submitted for publication to:
Annals of Surgery
Journal of Infectious Disease
Journal of Trauma
Infection and Immunity

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

burn	immunoglobulins	septicemia	Pseudomonas aeruginosa
trauma	opsonin	bacteria	Staphylococcus aureus
injury	anaerobe	infection	Escherichia coli
complement	phagocytosis	lipopolysaccharide	Proteus mirabilis
	opportunist	lipid A	Bacteroides fragilis

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Reduction in C3 conversion in patients with severe thermal injury was shown to be caused by a deficiency of proteins required for alternative complement pathway activation, rather than to an inhibitor of C3 conversion. No correlation was demonstrated between this humoral abnormality and the occurrence, duration, or outcome of septicemia. Consumption of the classical complement pathway occurred preferentially prior to and during septicemia in thermally injured patients. Reduction in classical pathway activity was not demonstrated in non-septic burned patients, suggesting that this humoral abnormality (cont'd.)

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

Unclassified

083800

20. (Cont.)

was predictive of septic episodes. Alternative pathway consumption occurred infrequently during septicemia and appeared to result from generation of C3b via consumption of the classical pathway. Consumption of components of the alternative and/or classical complement pathway did not decrease the opsonic capacity of the patients' sera for their own infecting microorganisms.

Multiple abnormalities of the classical and alternative complement pathways, immunoglobulins, and opsonins were shown to occur following abdominal trauma, some of which persisted after the first week post trauma. The humoral abnormalities in the trauma patients were not related to surgical procedures or to fluid imbalances. Patients who subsequently developed microbial infections were the only patients who had decreased classical pathway activity that appeared to result from consumption of components. Reduction in IgM occurring during the initial post trauma period was not found to be related to splenectomy.

A heterogeneity in the requirements for immunoglobulin and the alternative and classical complement pathways for phagocytosis and intracellular killing of clinical isolates of E. coli, P. mirabilis, K. pneumoniae, and S. marcescens by human PMNs was demonstrated. Strains isolated from burned patients did not demonstrate a unique pattern of opsonic requirements, in comparison to the same species isolated from other sources. The primary role of immunoglobulin in the opsonic process was shown to be for steps other than complement activation.

Purified lipid A and LPS were shown to activate terminal complement components in normal human or guinea pig sera equally efficiently. Other heat-stable components on the bacterial cell surface also appeared to be involved in complement activation. LPS was shown to activate the alternative and classical complement pathways, whereas lipid A activated only the classical pathway.

Immunoglobulin as well as components of the alternative complement pathway were shown to be required for phagocytosis and intracellular killing of B. fragilis and B. thetaiotaomicron by human PMNs. Neither IgG nor IgM acted alone to promote phagocytosis and intracellular killing of the Bacteroides strains by PMNs in the presence of complement. IgA did not participate in phagocytosis and intracellular killing of the Bacteroides strains. Immunoglobulin was not shown to be required for alternative pathway activation by the Bacteroides strains, and therefore must be required for other steps in the opsonic process. Encapsulated and non-encapsulated strains of Bacteroides interacted identically with PNHS and PMNs in vitro, suggesting that capsular polysaccharide was not a determining factor in host defense against these microorganisms. Although the lipopolysaccharide of B. fragilis was shown to be capable of activating the classical as well as the alternative complement pathway, protein or some other heat-labile component on the surface of Bacteroides appeared to be equally if not more important in initiating complement activation.

Strains of C. albicans isolated from normal human flora were shown to be phagocytosed and killed intracellularly by human PMNs in the presence of pooled normal human serum, whereas strains isolated from burned patients or medical patients were resistant to the bactericidal activity of the PMNs.

LEVEL II AD _____

REPORT #3

"Host Defense Against Opportunist Microorganisms Following Trauma"

ANNUAL SUMMARY REPORT

Ann B. Bjornson, Ph.D.
William A. Altemeier, M.D.
H. Stephen Bjornson, M.D., Ph.D.

JUNE, 1978

Supported by

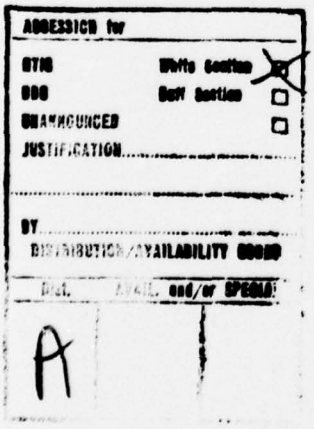
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

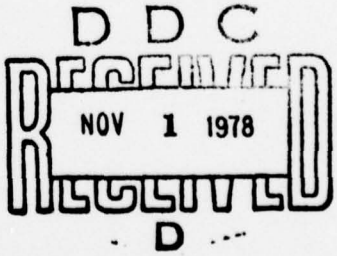
Fort Detrick,
Frederick, Maryland 21701

Contract No. DAMD-17-76-C-6023

University of Cincinnati

Cincinnati, Ohio 45221





"Approved for public release; distribution unlimited."

"The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents."

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal, Resources, National Academy of Sciences - National Research Council.

ACKNOWLEDGEMENT

The investigators express their gratitude to Dr. Clark West, Children's Hospital Research Foundation, Cincinnati, Ohio for reference sera and antisera to various complement proteins and to Dr. Michael Frank, National Institutes of Health, Bethesda, Maryland, for the C4 deficient guinea pigs. We also wish to thank Ms. Mary Cannon for the excellent technical preparation of this report.

ABSTRACT

Reduction in C3 conversion in patients with severe thermal injury was shown to be caused by a deficiency of proteins required for alternative complement pathway activation, rather than to an inhibitor of C3 conversion. No correlation was demonstrated between this humoral abnormality and the occurrence, duration, or outcome of septicemia. Consumption of the classical complement pathway occurred preferentially prior to and during septicemia in thermally injured patients. Reduction in classical pathway activity was not demonstrated in non-septic burned patients, suggesting that this humoral abnormality was predictive of septic episodes. Alternative pathway consumption occurred infrequently during septicemia and appeared to result from generation of C3b via consumption of the classical pathway. Consumption of components of the alternative and/or classical complement pathway did not decrease the opsonic capacity of the patients' sera for their own infecting microorganisms.

Multiple abnormalities of the classical and alternative complement pathways, immunoglobulins, and opsonins were shown to occur following abdominal trauma, some of which persisted after the first week post trauma. The humoral abnormalities in the trauma patients were not related to surgical procedures or to fluid imbalances. Patients who subsequently developed microbial infections were the only patients who had decreased classical pathway activity that appeared to result from consumption of components. Reduction in IgM occurring during the initial post trauma period was not found to be related to splenectomy.

A heterogeneity in the requirements for immunoglobulin and the alternative and classical complement pathways for phagocytosis and intracellular killing of clinical isolates of E. coli, P. mirabilis, K. pneumoniae, and S. marcescens by human PMNs was demonstrated. Strains isolated from burned patients did not demonstrate a unique pattern of opsonic requirements, in comparison to the same species isolated from other sources. The primary role of immunoglobulin in the opsonic process was shown to be for steps other than complement activation.

Purified lipid A and LPS were shown to activate terminal complement components in normal human or guinea pig sera equally efficiently. Other heat-stable components on the bacterial cell surface also appeared to be involved in complement activation. LPS was shown to activate the alternative and classical complement pathways, whereas lipid A activated only the classical pathway.

Immunoglobulin as well as components of the alternative complement pathway were shown to be required for phagocytosis and intracellular killing of B. fragilis and B. thetaiotaomicron by human PMNs. Neither IgG nor IgM acted alone to promote phagocytosis and intracellular killing of the Bacteroides strains by PMNs in the presence of complement. IgA did not participate in phagocytosis and intracellular killing of the Bacteroides strains. Immunoglobulin was not shown to be required for alternative pathway activation by the Bacteroides strains, and therefore must be required for other steps in the opsonic process. Encapsulated and non-encapsulated strains of Bacteroides interacted identically with PNHS and PMNs in vitro, suggesting that capsular polysaccharide was not a determining factor in host defense against these microorganisms. Although the lipopolysaccharide of B. fragilis was shown to be capable of activating the classical as well as the alternative complement pathway, protein or some other heat-labile component on the surface of

Bacteroides appeared to be equally if not more important in initiating complement activation.

Strains of *C. albicans* isolated from normal human flora were shown to be phagocytosed and killed intracellularly by human PMNs in the presence of pooled normal human serum, whereas strains isolated from burned patients or medical patients were resistant to the bactericidal activity of the PMNs.

Table of Contents

Index of Figures

Index of Tables

I. INTRODUCTION

A primary cause of morbidity and mortality in military personnel who have sustained burns, gunshot and high explosive wounds, or crush injuries is microbial infection (1,2). The widespread prophylactic use of antibiotics has not only failed to decrease the incidence of infection, particularly in the burned patient, but has also contributed to the complexity of the problem through the development of infections caused by antibiotic resistant microorganisms. In our medical center, Pseudomonas aeruginosa, Proteus, Escherichia coli, Staphylococcus aureus, and Candida albicans are the microorganisms which are most frequently associated with septic complications in the thermally injured patient, and Bacteroides fragilis has assumed a major role in the etiology of peritonitis caused by penetrating abdominal injury. The increased incidence of recovery of penicillin resistant and more recently, clindamycin resistant strains of B. fragilis (3) suggests that these microorganisms may become one of the major infectious problems of the future.

Management of surgical infections has classically involved antibiotic therapy and the meticulous care of the surgical wound. However, in addition to these therapeutic modalities, attention has recently been focused upon defining abnormalities of host defense mechanisms which may predispose the injured patient to microbial infection. Studies of alterations of host defense mechanisms in surgical patients have predominantly been carried out in patients with severe thermal injury. The data obtained from these investigations have provided evidence to suggest that neutrophil anti-staphylococcal activity (4), phagocytic function of the reticuloendothelial system (5), cell-mediated immunity (6), serum opsonins (7-9), levels of immunoglobulins (10-14), and classical (9,15) and alternative (8,9) complement components are reduced following burn trauma.

The investigation described in this report was undertaken to determine the cause and significance of the various alterations of complement in burned patients. In addition, studies were initiated to determine the occurrence, duration, and significance of the humoral abnormalities in patients with non-burn trauma which we have recently documented. Experiments were also conducted to provide basic information to increase our understanding of the humoral host defense mechanisms which are operative against the opportunist microorganisms which cause serious infections in injured patients.

II. BACKGROUND

A. Changes in Humoral Components of Host Defense in Patients Following Burn Injury

1. Studies to determine the association between changes in serum factors and septicemia in burned patients

In our previous studies, reduction in the immunochemical levels and functional activities of components of the classical and alternative complement pathways was associated with septicemia in two burned patients (9). Reduction in the classical complement pathway occurred during the first 10 days postburn in an infant who developed septicemia with S. aureus on the third postburn day which persisted through the sixth day. Reduction in both the classical and alternative complement pathways was also observed in a 39 year old female who had multiple episodes of Pseudomonas septicemia and candidemia during her clinical course and who died of septic shock. The investigation to be described was undertaken to determine if these preliminary data could be substantiated and to determine if complement consumption reduced the opsonic capacity of the patient's serum for his own infecting microorganism (16).

Fifteen patients with severe thermal injury were followed for up to 9 weeks postburn. Patients who appeared to be at the greatest possible risk of infection because of burn size or age or both were selected for the study. Serum samples were obtained from the patients as soon after the injury as possible and then at weekly intervals. In those patients who developed septicemia, serum samples were also obtained two additional times per week until blood cultures became negative. Blood cultures were drawn on all patients at least one time per week by our staff and additional blood cultures were drawn at the discretion of the attending physicians. This procedure was adopted for the purpose of documenting negative as well as positive cultures obtained on the patients. Septicemia was documented by clinical findings and positive blood cultures. The clinical criteria used for the diagnosis of septicemia were (a) chills and fever, (b) tachycardia, (c) hypotension, and (d) disorientation.

Five of the fifteen patients had one or more positive blood cultures and clinical signs of septicemia during their clinical course. The data indicated that consumption of the classical complement pathway was associated with and was probably caused by septicemia in the thermally injured patients. In three of five septic burned patients, decrease in the functional activity and immunochemical levels of components of the classical complement pathway occurred prior to and during septic episodes. Both of the other two patients also had decreased classical pathway activity prior to the development of septicemia. In one of the patients, classical pathway activity was decreased during the first septic episode, but not during the second episode. In the other patient, classical pathway activity was decreased during the second septic period but not for the duration of the first episode. The results obtained in the patients can probably be at least partially attributed to the administration of blood products. The later patient received whole blood and single donor plasma during the first but not the second septic episode. The other patient whose classical pathway activity was reduced

during the first but not the second septic episode, received equivalent amounts of single donor plasma per day during the two time intervals but twice the amount of whole blood per day during the second septic episode.

One of the patients in our study, who was bacteremic, served as an excellent control for determining the relationship between changes in complement and septicemia. No changes in the classical or alternative complement pathways occurred prior to or on the day that this patient's blood culture was positive and no clinical signs of septicemia were documented. An early reduction in classical pathway activity was demonstrated in all of the septic patients during the initial postburn period, suggesting that this humoral abnormality may be predictive of a septic episode and may possible predispose the patient to infection. Our results tend to rule out the possibility that the initial reduction in classical pathway activation occurring during the first week postburn resulted from infection, since blood cultures were consistently negative in all but one of the patients during this time.

The classical pathway appeared to be activated preferentially in the burned patients during septic episodes. Although C3 conversion by inulin was often reduced during septic episodes, levels of factor B were generally normal or elevated. Since it is well known that factor B is consumed during alternative pathway activation, the results suggested that C3 conversion via the alternative pathway was reduced in the burned patients due to inhibition rather than to activation and thus consumption of alternative pathway components. Inhibition of alternative pathway activation or blocking of activation due to a deficiency of an alternative pathway component would provide an explanation for the observed preferential activation of the classical pathway. Further studies regarding evidence for presence of a circulating inhibitor of C3 conversion via the alternative pathway in burned patients is presented in the next section of this report. This topic is also discussed in section C1 of this report dealing with preferential utilization of the classical pathway in burn sera during opsonization of E. coli 075.

In only one patient did consumption of components of the classical complement pathway occurring during septicemia decrease the opsonic capacity of the patient's sera for her own infecting microorganism, an isolate of E. coli; sera from the same patient which could not opsonize E. coli, opsonized her infecting strain of S. aureus normally. The microorganisms which were isolated from the other septic burned patients and used to test the opsonic capacity of the patients' sera were also, with one exception, strains of Staphylococci. Since there is evidence that S. aureus can be opsonized by normal IgG in the absence of complement (17,18), the lack of demonstration of reduction in the opsonic capacity of the patients' sera for their infecting strains of Staphylococci might be related to the lack of requirement

for complement for opsonization of the strains. It will be very important in future studies to determine if reduction in serum opsonic activity occurs for certain bacteria and not for others as was observed in this group of septic patients.

It should be emphasized that the E. coli isolate as well as the strains of Staphylococci and S. faecalis isolated from the burned patients were not susceptible to direct lysis by pooled normal serum in the absence of leukocytes or to phagocytosis and intracellular killing by normal leukocytes in the absence of serum. In addition, the concentration of serum and incubation periods used in the opsonic assays were specific for each infecting microorganism. Concentrations of the patients' sera were based on the minimal amount of pooled normal human serum which was found to promote maximal intracellular killing of the microorganism by normal leukocytes during the shortest incubation period.

Another interesting observation which was derived from this study was that strains of C. albicans isolated from the burned patients were not phagocytosed and killed intracellularly by normal leukocytes in the presence of five to ten times the concentration of normal human serum required for phagocytosis of other microorganisms. Further studies will be initiated to determine if the C. albicans strains isolated from the burned patients are more resistant to opsonization by normal serum than strains of C. albicans isolated from other sources and to determine the human serum proteins required for opsonization of the C. albicans strains.

Data from the burned patients who did not develop septicemia was grouped according to burn size to enable comparisons to be made between the present study group and previously studied patients (9). The only difference which was observed in the data from the groups of burned patients in this study and our previously studied groups of patients with similar burn sizes was in the results of the analyses of C3 conversion by inulin in patients with the largest burn sizes. In the present study, C3 conversion by inulin was markedly reduced initially and remained reduced for the duration of the study. In the previous study, C3 conversion was not reduced until after the first ten days postburn. However, in our original work demonstrating reduction in C3 conversion in the sera of patients with severe thermal injury, C3 conversion in the sera of some of the patients was reduced during the initial ten day postburn period and in others it was not (8). The discrepancy in the C3 conversion results remains to be explained, however it does not appear to be related to burn size.

2. Studies to determine the mechanism of reduction in C3 conversion via the alternative pathway in burned patients

Our previous studies showed that conversion of C3 by inulin in sera from severely burned patients was reduced after the first 10 days postburn and was normalized by the seventh week (8,9). The occurrence and duration of the reduction in C3 conversion were found to

be directly related to the severity of the burn injury. Reduction in the C3 converting activity of the burn sera could not be fully restored to normal by addition of 50% pooled normal human serum, providing preliminary evidence that this complement abnormality was caused by an inhibitor. An investigation was therefore undertaken to test directly the hypothesis that reduction in C3 conversion by inulin in the burn sera was caused by a circulating inhibitor.

The data did not exclude the possibility that reduction in C3 conversion by inulin in the burn sera was caused by a deficiency of critical normal serum proteins required for C3 conversion. However, they provided preliminary support for the concept that this complement abnormality was caused by an elevation of a normal regulatory protein. The serum protein was present in the euglobulin fraction and appeared to be present in greater concentration in burn sera than in normal sera.

The regulatory protein is probably not C3b inactivator (C3b INA) which inactivates C3b thereby inhibiting the amplification loop of the alternative pathway formed by the C3 convertases, C3b,B or C3b,B,P (19-21). Quite recently a new regulatory protein, βIH, has been discovered (22,23). βIH potentiates the inactivation of C3b by C3b INA and, in addition, directly inhibits C3b and the activity of the alternative pathway convertases C3b,B and C3b,B,P (24). The protein has been isolated from normal human plasma and shown to be an asymmetric molecule with a molecular weight of 300,000 daltons. The protein is a euglobulin which is distinct from C3b INA. Our future studies will be directed toward determining if reduction in C3 conversion in burn sera is caused by an elevation of βIH or another as yet undescribed regulatory protein of the alternative pathway or by a deficiency of a protein or proteins required for alternative pathway activation.

B. Changes in Humoral Components of Host Defense in Patients with Non-Burn Trauma and in Septic Patients without Trauma

In our previous studies (8,9), and in the studies cited in section A of this report, patients with burn injury were found to have multiple alterations of complement and immunoglobulins. Some of the humoral alterations were demonstrated in the burned patients during septic episodes, whereas other alterations were clearly unassociated with systemic bacterial or fungal infection. An investigation was undertaken to determine if the changes in complement components and immunoglobulins which were demonstrated in septic and non-septic burned patients were unique to patients with burn injury or were also observed in patients with non-burn trauma or in septic patients without trauma. It was hoped that the information obtained from the investigation would help to establish the etiology of the humoral alterations in the burned patients and to provide new information regarding the integrity of host defenses in patients with non-burn trauma.

Abnormalities of both the classical and alternative complement pathways were found to occur immediately following severe blunt or penetrating abdominal trauma (25). Conversion of C3 by CoVF which is a functional measurement of the alternative pathway was reduced in the

sera of the trauma patients, and decrease in the level of properdin and KAF was also demonstrated in the trauma sera. CH_{50}, a functional measurement of the classical pathway, and the immunochemical level of C5 were also decreased. Conversion of C3 by inulin and levels of factor B,C1,C4,C2, and C3 were found to be normal in the patients' sera.

It is unclear why C3 conversion by CoVF was reduced in the trauma sera, whereas C3 conversion by inulin in the sera was normal. C3 conversion by inulin requires factors B, D, initiating factor, C3, and magnesium ions (19,21), and C3 conversion by CoVF requires factors B, D, magnesium ions, and an additional euglobulin termed factor E (26). It is possible that the sera from the trauma patients was deficient in factor E in addition to properdin and C3b INA. Further studies are required to determine the identity and significance of deficient or abnormally functioning alternative pathway components in the sera from trauma patients.

Reduction in C5 in the trauma sera could have resulted from decreased C3b INA. C3b INA inactivates C3b which is required for formation of the alternative pathway C5 convertase, C3b,B,P (19,20). A decrease in C3b INA would cause an increase in C3b which through formation of the C5 convertase would result in increased turnover of C5. Activation of C5 leads to consumption of C6 to C9, and therefore these components are probably also reduced in the trauma sera.

A decrease in serum IgM was also observed in the trauma patients. A concommitant reduction in serum IgM (27) and properdin (28) levels has previously been demonstrated in splenectomized children. Decrease in IgM level in patients with sickle cell disease has correlated well with splenic afunction as measured by spleen scanning (29). In addition, children who undergo splenectomy for various hemolytic diseases or for traumatic rupture of the spleen have decreased IgM levels, supporting the concept that there is a correlation between splenic mass and concentration of this immunoglobulin (30). The reduction in IgM concentration does not appear to be the result of decreased splenic IgM synthesis, since IgM synthesis could be adequately assumed by other reticuloendothelial and lymphoid tissue. All of our patients had splenic trauma and all of those that survived subsequently underwent splenectomy. Thus, the decrease in IgM concentration in the sera of our patients may have been related to spleen damage. Our future studies will be designed to determine the correlation between spleen function and IgM level in the trauma patients in an attempt to determine the mechanism of the reduction in the level of this immunoglobulin. In addition, it will be of interest to determine the relationship between splenic function and reduction in properdin, KAF, and C5 in the trauma patients.

There have been virtually no investigations into the sequential effect of trauma on humoral host defenses. In addition, there are few studies that provide experimental evidence that trauma does indeed

increase susceptibility to infection. Conolly et al. found significantly more infections in rabbits injected subcutaneously with P. aeruginosa and subjected to mechanical trauma to the thigh than control animals that did not receive this injury (31). Cuthbertson et al. (32) subjected patients with bone fractures to varying degrees of cold temperature, 20°C and 30°C respectively. Levels of IgG were the same in both groups, however some minor fluctuations in IgM level were observed during ten days in the group subjected to the lower temperature. The changes were not impressive, since all variations were within the normal range.

Studies on the effects of wound trauma on humoral antibody responses are also limited. Havens et al. (33) could detect no reduction in the antibody response to diphtheria toxoid in shick-negative wounded patients. Balch showed that the secondary response to tetanus toxoid was normal in trauma patients (34).

Trauma has been shown to depress clearance by the reticuloendothelial system (35-40), phagocytosis by neutrophils (41,42), and cell mediated immunity (43). These changes are of short duration lasting during the first few days after trauma and are followed by restoration of normal activity. The cause and significance of these cellular abnormalities is unknown.

It is obvious from our pilot study demonstrating changes in humoral components of host defense in trauma patients that further comprehensive studies on larger numbers of patients are needed. It will be important to determine the cause and significance of the humoral abnormalities which occur following trauma and to determine the duration of the abnormalities.

Our study did not identify any reduction in components of the classical or alternative complement pathways in septic patients without trauma, suggesting that complement consumption in the septic burned patients was a result of synergism between the infection and the trauma. McCabe (44) previously showed that C3 levels were decreased in medical patients with septic shock, as compared to C3 levels in patients with uncomplicated bacteremia. The frequency of occurrence of shock or fatal outcome paralleled the degree of lowering of C3 levels in the patients. Fearon et al. (45) subsequently demonstrated decreased immunochemical levels of factor B, properdin and C3,C5,C6, and C9 in patients with septic shock in comparison to those patients with uncomplicated bacteremia, suggesting that consumption of complement occurred via the alternative pathway. Mean levels of classical components C1,C4, and C2 in the bacteremic patients in whom shock subsequently developed did not differ from those in patients with uncomplicated bacteremia.

Although none of our medical septic patients was hypotensive at the time of serum sample collection, 4 of the 10 patients died of septic shock on the following day, and no complement consumption was

demonstrated in these patients. In addition, consumption of the classical complement pathway was demonstrated in septic burned patients who were not hypotensive and survived. These observations suggested that classical complement pathway consumption associated with septicemia is unique to the thermally injured patients.

C. Normal Human Serum Opsonins for Opportunist Microorganisms

1. Studies to determine the mechanisms of complement activation and the role of immunoglobulin in opsonization of E. coli 075 and other aerobic opportunist microorganisms

In our previous studies, the classical complement pathway was shown to be utilized exclusively in burned patients during opsonization of E. coli 075. Serum opsonic activity for E. coli 075 and classical pathway activity were decreased concurrently in the burned patients during the first week postburn, despite normal alternative pathway activity as measured by C3 conversion by inulin. This observation led us to initiate studies to answer two important questions related to the definition of serum proteins required for opsonization of E. coli 075 and to alterations of these humoral factors in burned patients. The questions were as follows: (1) Was the classical pathway utilized in the burn sera during opsonization of E. coli 075 because the alternative pathway could not be activated, or was the classical pathway utilized preferentially?; (2) Why was the switch to an apparently functional alternative pathway and thus normal opsonization not possible, when classical pathway activity was decreased in the burn sera?

Our initial experimental studies to answer these questions were directed toward determining if E. coli 075 was, in fact, capable of activating the alternative complement pathway. The lipopolysaccharide (LPS) portion of the cell wall of gram-negative bacilli, such as E. coli 075, is known to be responsible for its anticomplementary activity (46-48). The moiety of the LPS which is responsible for complement activation is controversial. There is some experimental evidence to indicate that it is the lipid A moiety of the LPS which is responsible for this biological activity (49,50). Because E. coli 075 might be unique in the mechanism of complement activation it was capable of initiating, we also investigated the ability of other representative gram-negative bacilli and a gram-positive opportunist, S. aureus, to activate the alternative complement pathway. In addition, we included S and R forms (Rb and Re) of S. minnesota containing varying amounts of the polysaccharide portion of the LPS in an attempt to determine the moiety of the cell wall which was responsible for alternative pathway activation. S. minnesota S form contains a complete LPS, the Rb mutant lacks the O antigen and acetylglucosamine attached to the terminal glucose but contains the rest of the basal core, and the Re mutant contains only KDO and lipid A.

The data obtained from our investigation suggested that the classical pathway was utilized in the burn sera during opsonization of E. coli 075 because alternative pathway proteins required for

opsonization of this microorganism were not functioning properly. This conclusion was based on several pieces of experimental evidence as follows: (a) Intact cells of E. coli 075 were found to be capable of activating the alternative complement pathway. (b) Preliminary experiments using S. minnesota cell wall mutants indicated that the polysaccharide portion of the LPS was responsible for alternative pathway activation, whereas the lipid A moiety was responsible for activation of the classical pathway. These results require confirmation utilizing purified LPS and lipid A prepared from E. coli 075. However, they suggested that microorganisms such as E. coli 075 with complete polysaccharide regions in their cell walls would activate the alternative pathway under normal conditions, since this region of the gram-negative cell wall is outermost. The lipid A moiety is the innermost portion of the lipopolysaccharide and is located directly next to the mucopeptide layer of the cell wall. The classical pathway would therefore not be activated unless the cell wall was altered in some manner as to expose the lipid A or unless the alternative pathway was unable to be utilized. (c) Heat-stable immune IgG antibodies to E. coli 075 which would normally trigger classical pathway activation were not demonstrated in either normal or burn sera. In fact, the data suggested a minimal role, if any, for immunoglobulin in opsonization of E. coli 075. These later observations confirmed the results of Jasin who also demonstrated that opsonization of E. coli 075 proceeded normally in immunoglobulin depleted human serum (17).

The tentative conclusion that alternative pathway proteins required for opsonization of E. coli 075 were abnormal in the burn sera requires confirmation by experimental studies. This possibility is extremely interesting, however, in light of our previous observation that C3 conversion by inulin in the burn sera was normal when E. coli opsonization and classical pathway activity were reduced. Inulin is a recognized activator of the alternative pathway, and all of the studies which are currently available on the protein interactions of the alternative pathway have utilized zymosan or inulin as the activating substance. Our data would indicate that when bacteria, such as E. coli 075, are used as the activating substances, the protein interactions involved in alternative pathway activation may differ from the protein interactions involved when inulin is used as the activating substance. The sequence of recognized protein interactions may be different or proteins in addition to factors B, D, and initiating factor may be required to generate an alternative pathway C3 convertase by the bacteria. These possibilities emphasize the importance of future studies to determine the identity and sequence of proteins required for opsonization of gram-negative aerobes such as E. coli 075.

Our study also showed that gram-negative aerobic bacilli, in addition to E. coli 075, were capable of activating the alternative pathway. Efficient alternative pathway activation was achieved utilizing intact cells of P. aeruginosa, P. mirabilis, and S. minnesota S form. S. aureus, a microorganism which frequently causes serious infections in burned patients, was not found to be as efficient as the gram-negative bacilli in activating the alternative pathway, although this microorganism appeared highly active in initiating classical pathway activation.

The data obtained in this investigation have also aided in the interpretation of some of our unexplained previous observations regarding changes in humoral factors in burned patients. In this regard, inhibition of formation of an alternative pathway C3 convertase which we have demonstrated in burned patients may be of critical importance to the patient if classical pathway activity is decreased. Our results suggest that each complement pathway should be able to compensate for the other, if either is not functioning properly. If the alternative pathway is unable to be activated due to an inhibitory factor and classical pathway components are consumed during septicemia, then opsonization of the patient's sera for his infecting microorganism should be reduced if the organism requires complement activation for opsonization. Our results presented in section A1 of this report support this thesis.

Another interesting possibility which is supported by this investigation is that there may be more than one way to activate the classical pathway. It is well known that immune IgG antibacterial antibodies can bind the C1q portion of C1 and thereby initiate the rest of the classical sequence. Our results support the concept that classical pathway activation can also occur in the absence of antibody. Loos et al. (50) and Morrison and Kline (51) have provided evidence to suggest that the lipid A region of the LPS molecule interacts directly with C1 to initiate classical pathway activation by a mechanism which does not require antibody. Quite recently, Verhoeff et al. (52) had shown that S. aureus 502A requires the classical pathway for optimal phagocytosis by human leukocytes without the participation of immunoglobulin.

2. Studies to determine the requirement for immunoglobulin and complement in opsonization of B. fragilis and B. thetaiotaomicron

In our previous studies, methodology was developed for measuring the in vitro interaction of human leukocytes and serum factors in phagocytosis and intracellular killing of gram-negative non-sporulating anaerobes (53). Strains of Bacteroides fragilis and Bacteroides thetaiotaomicron were shown to be killed by human leukocytes in the presence of pooled normal human serum, but not by either leukocytes or serum alone. The investigation to be described was undertaken to define the role of immunoglobulin and complement in phagocytosis and intracellular killing of these anaerobic strains by leukocytes. All of the experiments to be described were carried out under anaerobic conditions as previously detailed (53).

Evidence was provided to indicate a requirement for immunoglobulin and components of the alternative complement pathway in normal human serum for opsonization of B. fragilis and B. thetaiotaomicron. This represents a totally new observation, since there is currently no literature available regarding the definition of serum proteins required for opsonization of these gram-negative non-sporulating anaerobes.

The experimental data in support of this preliminary conclusion are as follows: (a) Human sera depleted of immunoglobulin, C3, factors B or D or terminal complement components C3 to C9 did not support phagocytosis and intracellular killing of the microorganisms by normal human

leukocytes. (b) The opsonic activity of human serum heated at 50°C for 30 minutes for the strains was restored to normal by highly purified factor B, but not by human C2, the component of the classical pathway which is labile at 50°C. (c) Properdin depleted human serum supported normal phagocytosis of B. fragilis and only partially supported phagocytosis of the B. thetaiotaomicron, suggesting participation but not an absolute requirement for properdin for opsonization of the B. thetaiotaomicron. This observation is not surprising, since C3 conversion and thus presumably opsonization can occur in the absence of properdin. The function of properdin is to stabilize the C3 convertase, C3b,B formed by the feedback mechanism involving factors B, D, and C3b (19,20).

In our investigation, immunoglobulin in addition to components of the alternative complement pathway was found to be required for opsonization of both Bacteroides strains. Available information regarding the role of immunoglobulin in opsonization of microorganisms is controversial. Antibody of the IgG class, in addition to components of the alternative complement pathway, has been shown to participate in opsonization of P. aeruginosa (54,55) and Streptococcus pneumoniae (56). In contrast, opsonization of strains of E. coli (17), S. epidermidis, S. aureus, S. marcescens, S. viridans, and S. faecalis (57) has been shown to proceed normally in hypogammaglobulinemic sera.

The question of the role of immunoglobulin in activation of the alternative pathway is also unanswered. Available evidence indicates that activation by inulin or zymosan occurs in the absence of immunoglobulin (19). In addition, lysis of Trypanosoma cyclops (58) and rabbit erythrocytes (59) by normal human serum has been shown to require activation of the alternative pathway without an apparent requirement for conventional antibodies. On the other hand, antibody of the IgG class has been shown to participate in alternative pathway mediated lysis of measles virus infected cells (60) as well as in opsonization of bacteria as described above.

Since complement activation is required for opsonization of bacteria, one of the most important questions in this research area remaining to be answered is whether immunoglobulin is required directly for activation of the alternative pathway or for binding of the bacterium to receptors on the leukocyte cell membrane surface. The experimental design of our future studies will be focused on answering this as yet unresolved issue.

The results of our investigation indicated that the human serum proteins required for opsonization of B. fragilis and B. thetaiotaomicron were similar if not identical. This finding raises the question as to the importance of the polysaccharide capsule to the virulence of B. fragilis. Kasper has suggested that the polysaccharide capsule found almost exclusively on B. fragilis may act as an antiphagocytic agent (61). Our results would tend to support the thesis that the

antiphagocytic cell surface component is not unique to B. fragilis and probably may not be the primary virulence factor of this microorganism. Alternatively, our strain of B. thetaiotaomicron may be encapsulated although this possibility is considered unlikely, since Kasper has only found one of the several strains of B. thetaiotaomicron to be encapsulated. Our future studies will attempt to examine the opsonic requirements for American type culture collection isolates of B. fragilis and B. thetaiotaomicron used by other investigators. Of additional importance will be to examine the interaction of human serum and leukocytes with strains of B. vulgatus and B. distasonis, since none of these Bacteroides strains has been shown to contain capsular material.

III. EXPERIMENTAL APPROACH

Studies in three related areas of research were proposed for this application. Each research area was to be pursued in greater depth than in our previous proposals. Our primary area of research was to continue to be the evaluation of changes in humoral components of host defense following burn trauma. This area was to include a continuation of studies to determine the effects of septicemia on complement activity and opsonic function, and the mechanism and significance of reduction in C3 conversion by inulin. In addition, comprehensive studies were to be initiated to further evaluate the integrity of the alternative complement pathway in the burned patients, since in our previous studies only one parameter, C3 conversion by inulin, was used for determining the functional activity of this pathway. The second research area was to be focused on patients with types of trauma other than burn injury. The research which was proposed was to include studies to determine the identity, duration, and significance of changes in the alternative and classical complement pathways in patients with abdominal trauma, and studies to determine if the changes in humoral factors were unique to patients with abdominal trauma or were also observed in patients with other types of nonburn trauma. The third area of research to be pursued was to deal with humoral host defense mechanisms against the opportunist microorganisms which cause serious microbial infections in trauma patients. This area was to include a continuation of studies to determine the requirements for immunoglobulin and components of the alternative complement pathway for opsonization of B. fragilis and B. thetaiotaomicon, and of studies to identify the moieties of the cell wall of gram-negative aerobic and anaerobic bacilli which are responsible for activation of the alternative and classical complement pathways. In addition, studies were to be initiated to determine the role of immunoglobulin in opsonization of these microorganisms and the in vitro interaction of human serum and leukocytes with nonencapsulated species of Bacteroides. Also, studies were to be undertaken to compare the human serum opsonic requirements for strains of C. albicans isolated from burned patients in comparison to strains isolated from other sources.

Fifteen to 20 burned patients who were at the greatest possible risk of infection were to be selected for the study. Sera were to be collected from the patients one time per week for 6 to 8 weeks postburn. The alternative complement pathway was to be assessed by measuring C3 and C5 conversion by inulin and cobra venom factor (CoVF), functional activity of factors B and D and of the total alternative pathway, and the immunochemical levels of native C3, factor B, C3b INA, and properdin. Conversion of C3 in the burn sera by inulin and CoVF was to be measured by a hemolytic method as well as by reduction in the concentration of the B antigenic determinant of C3. Comparisons were to be made between C3 conversion by the activating substances in burn sera treated with EGTA and supplemented with magnesium ions, in which classical pathway activity is blocked, and in untreated burn sera. Classical pathway activity was to be assessed by measuring total hemolytic complement (CH_{50}) and immunochemical levels of C1q, C4, C2, C3, and C5. Septicemia was to be documented in the burned patients by positive blood cultures and clinical findings. If a patient developed septicemia, blood was to be drawn one or two additional times per week until the infection was cleared. The hypothesis that microbial infection perpetuates itself in the burned patient by causing complement consumption which reduces the opsonic capacity of the serum for the patient's infecting microorganisms was to be tested directly. The ability of the patient's sera to promote phagocytosis and killing of his infecting microorganism by normal human peripheral leukocytes was to be tested prior to and during septicemia, and, if the patient survived, after recovery. If more than one microorganism was isolated from a septic patient, each microorganism was to be tested separately as described above. Pertinent clinical information was to be recorded on each burned patient by a research nurse on an appropriate flow sheet. The information was to include the antibiotic regimen, culture data, clinical signs of sepsis, administration of blood products, and surgical procedures. The data was to be interpreted by Dr. S. Bjornson and Dr. Altemeier.

Experiments were to be performed to determine if reduction in C3 conversion via the alternative pathway was caused by elevation of a normal regulatory protein, by modification by burn injury of a normal euglobulin which did not under normal conditions regulate C3 conversion, by an inhibitor present in the euglobulin fraction of the burn sera produced as a result of burn injury, or by a deficiency of critical normal serum proteins required for C3 conversion. Immunochemical levels of βIH and C3b INA, regulatory proteins of the alternative pathway, and C3 conversion by inulin and CoVF were to be measured in serial serum samples obtained from ten burned patients. Conversion of C3 was to be measured both hemolytically and by reduction in the B antigenic determinant of C3 in EGTA treated magnesium supplemented burn sera and in untreated burn sera as described above. Regression analyses were then to be performed on the data to determine if a correlation existed between reduction in C3 conversion and elevation of βIH or C3b INA or when the two proteins were elevated simultaneously. In addition, euglobulin was to be prepared from both burn sera with reduced C3 conversion and from pooled normal serum. The euglobulin

fractions of the burn sera were to be added in increasing concentrations to normal human serum, and C3 conversion was to be measured and compared to C3 conversion in normal serum supplemented with euglobulin fractionated from pooled normal human serum. The euglobulin preparations which inhibited C3 conversion in normal serum were to be fractionated by QAE-Sephadex A-50, BioRex 70, and preparative isoelectric focusing. Active fractions were to be identified by their ability to inhibit C3 conversion in normal serum as described above. The chromatographic procedures which were to be selected for our study were to be used because comparisons could then be made between the inhibitory activity in the burn sera and the known inhibitors of C3 conversion in normal serum, i.e., βIH and C3b INA, since these procedures were used for the original separation of BIH and C3b INA. Preparative isoelectric focusing was to be used as the final purification step. If these procedures failed to isolate an inhibitor of C3 conversion in the burn sera, then studies were to be initiated to determine if reduction in C3 conversion in the burn sera was caused by a deficiency of serum proteins. Pooled normal human serum was to be fractionated by ion exchange and molecular sieve chromatography, and fractions were to be added to burn sera in an attempt to restore C3 conversion to normal.

The second area of research which was planned was a comprehensive study to assess the alternative and classical complement pathways, immunoglobulins, and serum opsonic activity in patients with nonburn trauma. The study was to involve three patient groups, i.e., patients with blunt or penetrating abdominal trauma, patients without trauma who had undergone appropriately matched abdominal surgical procedures, and patients with head injuries without abdominal trauma. The middle group of surgical patients without trauma were to serve as the control population. Ten patients in each group were to be followed for three weeks post admission. Serum samples were to be obtained as soon as the patients were identified and then two times per week for three weeks or until the patient was discharged if this occurred prior to three weeks. The alternative pathway was to be assessed by measuring C3 conversion by inulin and CoVF, total alternative pathway activity and functional activities of factors B and D, and immunochemical levels of native C3, factor B, properdin and C3b INA. Classical pathway activity was to be assessed by measuring CH_{50} and immunochemical levels of C1q, C4, C2, C3, and C5. Immunoglobulins G, A, and M were also to be quantitated in the patients' sera, since IgM was found to be markedly decreased initially following abdominal trauma in our preliminary study. Opsonic activity of the patients' sera for E. coli 075, S. aureus 502A, and P. aeruginosa 73044 were also to be determined. Opsonic activity of sera from our preliminary study group of ten trauma patients which had decreased alternative and classical pathway activity and decreased IgM were also to be measured using the microorganisms described above. The experiments were to be performed in an attempt to determine if the abnormalities decreased the opsonic capacity of the sera for microorganisms which frequently cause infections in trauma patients. Pertinent clinical information on the new study patients was to be recorded by the research nurse who was also

to obtain the specimens on these groups of patients. Of particular interest was to determine the association between humoral abnormalities and the occurrence of microbial infection in the patients with nonburn trauma and in the surgical patients without trauma.

The third area of research was to involve a continuation of studies to increase our understanding of humoral host defense mechanisms against the opportunist microorganisms which cause serious infections in trauma patients. Experiments were to be performed to provide further support for our previous preliminary observation that both immunoglobulin and components of the alternative complement pathway were required for opsonization of B. fragilis and B. thetaiotaomicron. Dose dependent restoration of the opsonic activity of immunoglobulin depleted, factor B depleted, and factor D depleted human sera were to be attempted using the respective purified human protein. In addition, studies were to be initiated to determine the normal human serum requirements for opsonization of B. distasonis and B. vulgatus. If it was demonstrated that these strains have the same opsonic requirements as B. fragilis and B. thetaiotaomicron, then a role for the capsule in host defense would be minimized, since the B. distasonis and B. vulgatus are nonencapsulated. Conversely, if it was shown that these strains were susceptible to complement mediated lysis or to phagocytosis and intracellular killing by leukocytes in the absence of serum, then the concept of an antiphagocytic effect of the capsule would have been strengthened. An integral part of these experiments was to be the demonstration of capsular material or lack of it on the strains of Bacteroides used in our study. Our experiments were to include several strains of each species.

Studies were also initiated to determine the moieties of the cell wall of gram-negative aerobic and anaerobic bacilli which activate the classical and alternative pathways, since this information would allow us to determine which pathway was activated preferentially during opsonization of the microorganisms under normal conditions. Lipid A and LPS were to be prepared from strains of E. coli, P. mirabilis, and P. aeruginosa isolated from the blood cultures of our burned patients. Lipid A and LPS were also to be prepared from E. coli 075, P. aeruginosa 73044, P. mirabilis 7056, and S. minnesota S form. In addition, Dr. Dennis Kasper (Harvard Medical School, Boston, Mass.) agreed to provide us with purified LPS, lipid, outer membrane protein and capsular polysaccharide from B. fragilis. These purified preparations and washed heat-killed bacterial cells from which they were derived were to be tested for their ability to activate the classical and alternative pathways. Classical and alternative pathway consumption were to be measured by comparing the functional activities of C1, C4, C2, C3, and C3 to C9 in human serum before and after incubation with each purified preparation or bacterial cell suspension. Alternative pathway activation was also to be measured by determining C3 to C9 consumption by the bacteria in C4 deficient guinea pig serum.

Studies were also to be performed to determine if immunoglobulin was required for opsonization of the bacterial strains by normal human serum because is was required for complement activation or for some other stage in the opsonic process. In this regard, there is no information currently available on the role of immunoglobulin in activation of the alternative complement pathway by bacteria. All of the available information on the role of immunoglobulin in activation of this pathway has been carried out utilizing zymosan or inulin as the activating substance, and a role for immunoglobulin in activation of the alternative pathway has not been demonstrated. The microorganisms which were to be used for these experiments were to be the clinical isolates of P. mirabilis, E. coli, and P. aeruginosa described above, as well as a strain of S. aureus isolated from the blood culture of a burned patient and the B. fragilis and the B. thetaiotaomicron used in our previous studies. The ability of hypogammaglobulinemia and normal sera to promote phagocytosis of the microorganisms by normal human leukocytes were to be compared. Those microorganisms which required more than a small amount of immunoglobulin for opsonization would not be phagocytosed in the presence of the hypogammaglobulinemic sera. In our previous studies, we have demonstrated that hypogammaglobulinemic sera did not support opsonization of the Bacteroides strains. We also have preliminary evidence to suggest that the sera also did not support opsonization of the E. coli, P. mirabilis and P. aeruginosa isolates. Washed cells of the test strains were to be incubated with the hypogammaglobulinemic and normal sera, and C3 to C9 consumption was to be measured. If the hypogammaglobulinemic sera could not support C3 to C9 consumption by the bacteria or opsonization of the bacteria, this observation would indicate that immunoglobulin was required for opsonization because it was required for complement activation. If the hypogammaglobulinemic sera supported normal C3 to C9 consumption by the bacteria but did not support opsonization of the bacteria, this observation would indicate that immunoglobulin was not required for complement activation but served some other function in the opsonic process.

Finally, experiments were to be conducted to determine if strains of C. albicans isolated from burned patients were more resistant to phagocytosis and intracellular killing by human leukocytes than C. albicans strains isolated from other sources. The ability of increasing concentrations of pooled normal human serum to promote intracellular killing of the strains by normal human leukocytes was to be determined. The strains of C. albicans to be tested were to include blood culture isolates from burned patients and isolates from clinical specimens obtained from other patient populations.

IV. PROGRESS REPORT

A. Changes in Humoral Components of Host Defense in Patients Following Burn Injury

1. Studies to determine the mechanism of reduction in C3 conversion in burned patients

a. Results

Our previous studies showed that conversion of C3 by inulin in sera from severely burned patients was reduced during the early acute burn phase and was normalized by the seventh week (8,9,16).The occurrence and duration of the reduction in C3 conversion were found to be directly related to the severity of the burn injury. Mixture of equal parts of burn sera with reduced C3 conversion and pooled normal human serum (PNHS) did not restore C3 conversion to normal. These preliminary results suggested that reduction in C3 conversion in the burn sera might be caused by a circulating inhibitor.

The hypothesis that reduction in C3 conversion in the burn sera was caused by an inhibitor was tested experimentally (Annual Summary Report, June 1977). Burn sera with normal or reduced C3 conversion were tested for their ability to inhibit C3 conversion by inulin in PNHS. None of the burn sera with normal C3 conversion inhibited C3 conversion to any extent when added in increasing concentrations to PNHS. Several of the burn sera with reduced C3 conversion inhibited C3 conversion when added to PNHS, whereas other burn sera with reduced C3 conversion had no inhibitory activity. When the data were subjected to statistical analysis, the differences which were observed between the burn sera with reduced C3 conversion and those with normal C3 conversion were not found to be significant. In addition, the values obtained when equal parts of burn sera and PNHS were added together were not considerably lower than those obtained when a complement deficient normal serum (PNHS heated at $56^{\circ}C$ for 30 minutes) was added to PNHS.

The inability to demonstrate statistically significant differences between the inhibitory activities of burn sera with reduced versus normal C3 conversion might have been related to the concentration of the inhibitor in sera with reduced C3 conversion. If the inhibitor was present in minimal amounts in the sera, then fractionation of the sera and concentration of the fractions should result in demonstration of inhibitory activity by one of the fractions. An alternative explanation for the data was that reduction in C3 conversion in the burn sera was caused by a deficiency of critical normal serum proteins required for C3 conversion. To test the first hypothesis, pseudoglobulin and euglobulin fractions of burn sera with reduced C3 conversion were added in increasing concentrations to PNHS, and C3 conversion by inulin was measured and compared to C3 conversion in PNHS supplemented with pseudoglobulin or euglobulin fractionated from PNHS. Euglobulin fractions prepared from either burn sera or PNHS inhibited C3 conversion by inulin when added to PNHS. However, the euglobulin fractionated from the burn sera was more inhibitory than the euglobulin fractionated from PNHS. Since recognized regulatory proteins of the alternative complement pathway C3b inactivator (C3b INA) and βIH are known to be euglobulins (22) the results described above provided preliminary support for the concept that reduction in C3 conversion in the burn sera might be caused by elevation of one or more of these proteins.

C3 conversion by inulin and immunochemical levels of C3b INA and β1H were measured in multiple samples of sera obtained from eight different burned patients. β1H determinations on the sera were performed in the laboratory of Dr. Shaun Ruddy, Medical College of Virginia, Richmond, Virginia. Clinical characteristics of the burned patients from which the sera were obtained (#1 to 8) are presented in Table 1. Utilizing single regression analysis, no correlation was demonstrated between C3b INA levels and C3 conversion by inulin in the burn sera (Figure 1). A correlation was also not demonstrated between β1H levels and C3 conversion by inulin in the burn sera (Figure 2). Multiple regression analysis with y equal to C3 conversion by inulin and x equal to β1H and C3b INA yielded an r value of 0.262 and a p value of 0.40. These results indicated that a simultaneous elevation of β1H and C3b INA also did not correlate with reduction in C3 conversion by inulin in the burn sera.

The results described above led us to hypothesize that the inhibitory activity of the burn euglobulin was unrelated to the original C3 converting of the burn sera from which it had been prepared, but could be explained by elevation of regulatory proteins. If this hypothesis was correct, then the inhibitory activity of euglobulin prepared from burn sera with normal C3 conversion should be equivalent to the inhibitory activity of euglobulin prepared from burn sera with reduced C3 conversion, providing C3b INA and β1H were elevated in the sera. Sera from Patients 5, 6, and 8 with normal C3 conversion and elevated levels of C3b INA and β1H were pooled, and each pooled serum was fractionated into pseudoglobulin and euglobulin. Each pooled serum (2 ml) was dialyzed against 0.008 M EDTA, pH 5.4, for 18 to 24 hours at $4^{o}C$. The euglobulin was deposited by centrifugation, and the pseudoglobulin was removed. The euglobulin was washed three times with 0.008 M EDTA and redissolved in 0.5 ml of 0.01 M Tris-HCl, pH 7.4, containing 0.3 M NaCl. The euglobulin and pseudoglobulin fractions of the sera were then dialyzed against 0.01 M phosphate buffered saline containing 5×10^{-4} M $CaCl_2$ and 1.5×10^{-4} M $MgCl_2$. PNHS was fractionated into pseudoglobulin and euglobulin fractions and dialyzed as described above for fractionation of the burn sera. The procedures for preparation of the euglobulin and pseudoglobulin fractions were identical to those used in our previous experiments.

Unfractionated pooled burn sera and the pseudoglobulin and euglobulin fractions of the pooled sera were added in increasing concentrations to PNHS, and C3 conversion by 10 mg/ml of inulin was measured and compared to C3 conversion in PNHS supplemented with pseudoglobulin or euglobulin fractionated from PNHS. The euglobulin fractions of the pooled burn sera inhibited C3 conversion by inulin in PNHS to a greater extent than the euglobulin fractionated from PNHS (Figure 3). These results indicated that the ability of the burn euglobulin to inhibit C3 conversion in PNHS was related to the concentration of regulatory proteins in the sera from which it had been prepared, but not to the original C3 converting activity of the sera. In addition, the results refuted the hypothesis that reduction in C3 conversion was caused by alteration by burn injury of a non-regulatory euglobulin to a configuration with regulatory activity.

The results of our initial experiments indicated that the C3 conversion values obtained when equal parts of burn sera with reduced C3 conversion and PNHS were added together were not equal to the C3 conversion values obtained when normal sera were added to PNHS. However, the C3 conversion values obtained when

Table 1. Clinical Characteristics of Patients with Burn Injury

Patient No.	Age	Sex[a]	Body Surface Injured[b]	
			Total %	Third Degree %
1	12	F	40	11
2	6	F	60	60
3	42	M	63	1
4	9	F	49	15
5	13	F	83	62
6	10	M	37	16
7	12	M	70	60
8	10	M	71	66
9	9	M	48	14
10	3	F	60	42
11	21	M	73	56
12	51	M	35	13
13	59	M	62	0
14	14	M	45	37
15	12	M	40	0
16	15	M	49	45
17	4	F	48	48
18	4	F	78	70
19	55	F	52	17
20	11	M	51	13
21	9	F	65	40
22	14	F	45	35
23	9	F	55	55

[a]M = male; F = female.
[b]All patients had flame burn injuries.

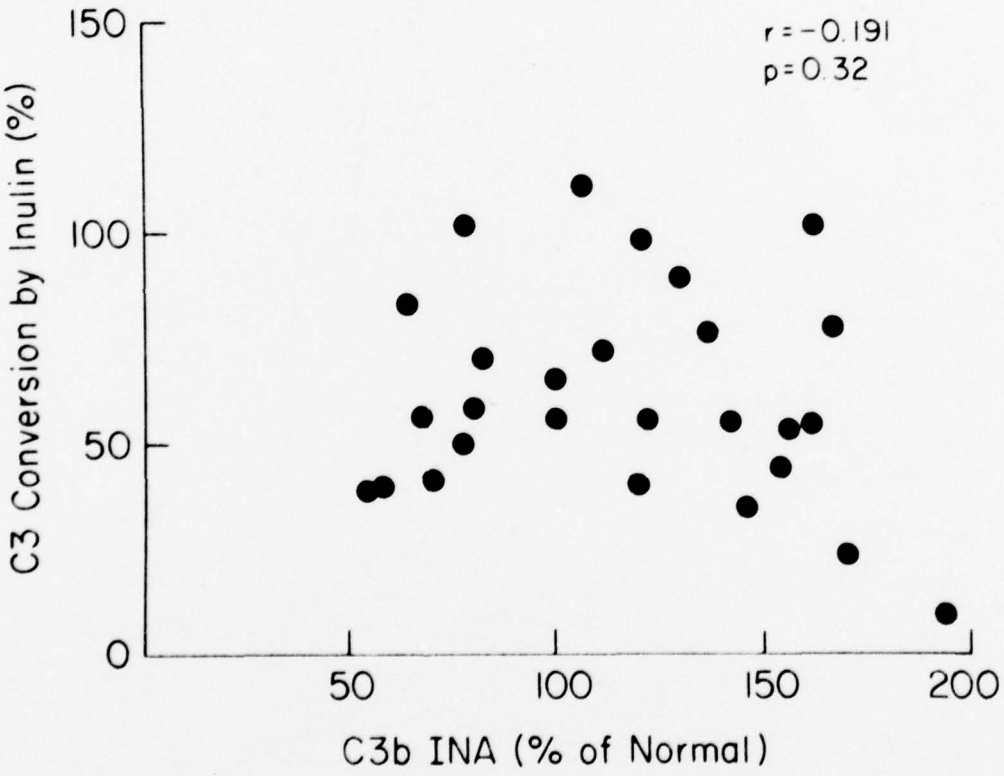


Figure 1. Relationship between immunochemical levels of C3b inactivator (C3b INA) and C3 conversion by inulin in multiple sera obtained from eight burned patients. The coefficient of linear correlation (r) was -0.191, and the p value was 0.32. The points represent the mean values of duplicate determinations.

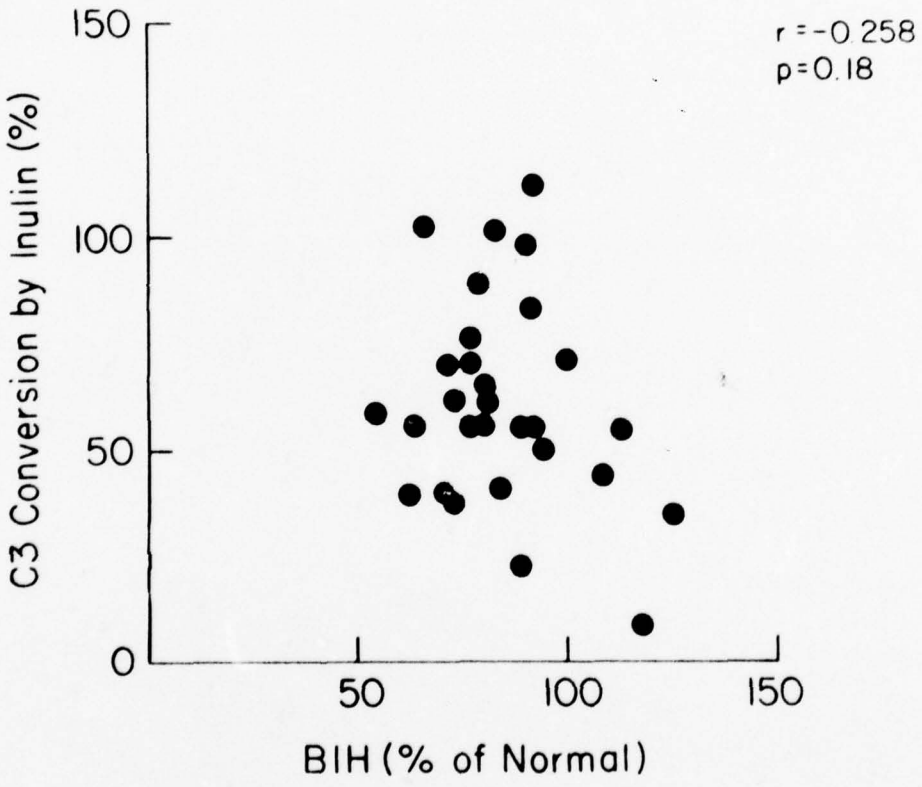


Figure 2. Relationship between immunochemical levels of βlH and C3 conversion by inulin in multiple sera obtained from eight burned patients. The coefficient of linear correlation (r) was -0.258, and the p value was 0.18. The points represent the mean values of duplicate determinations.

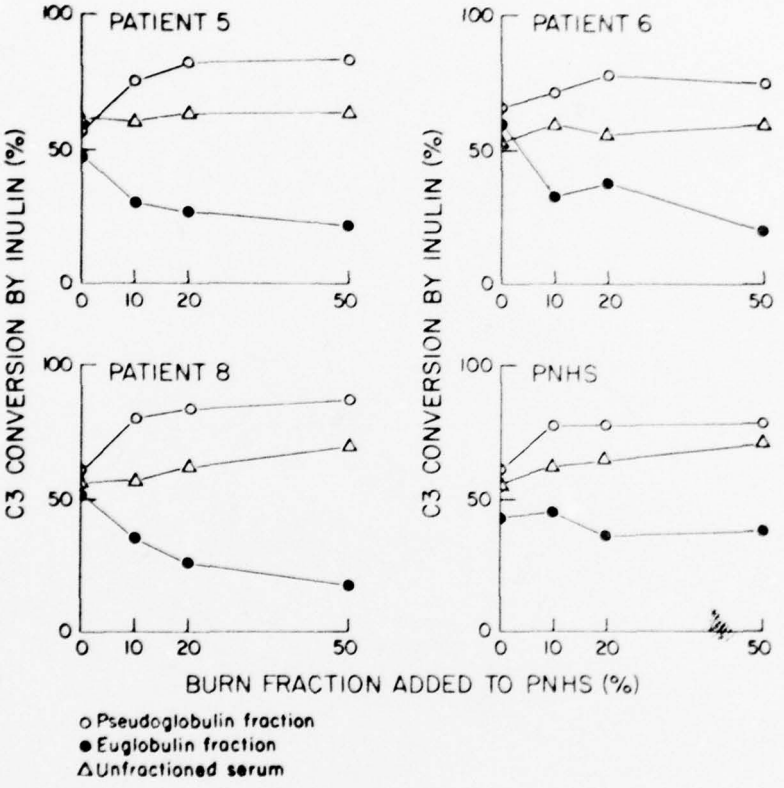


Figure 3. C3 conversion by inulin in PNHS supplemented with unfractionated burn sera and the euglobulin or pseudoglobulin fractions of pooled sera from Patients 5, 6, and 8. Sera collected on the following dates from the patients were pooled: Patient 5 - days 9, 33, and 40; Patient 6 - days 26, 32, and 39; and Patient 8 - days 10, 47, and 57. C3 conversion by inulin in the pooled burn sera prior to fractionation was 75% for Patient 5, 79% for Patient 6, and 82% for Patient 8. The points represent values of single determinations.

burn sera were added to PNHS were not significantly lower than those obtained when a complement deficient serum was added to PNHS. This observation led us to initiate a series of more extensive experiments on larger numbers of patients to determine the cause of reduction in C3 conversion and the relationship between reduction in C3 conversion and septicemia in burned patients.

Conversion of C3 by inulin was measured during 62 days postburn in the sera of nine patients (#8 to 16) who had no evidence of systemic microbial infection as documented by negative blood cultures (Figure 4). An additional population of seven patients (#17 to 23) with septicemia as documented by positive blood cultures and clinical findings was studied for 58 days postburn (Figure 5). Refer to Table 1 for the clinical characteristics of the patients. The patients fell into four groups as follows: (a) those with normal C3 conversion for the duration of the study (Patients 15, 21, 23); (b) those with abnormal C3 conversion for the entire study (Patients 10, 13); (c) those with normal C3 conversion initially which became abnormal during the study period (Patients 8, 9, 11, 12, 14, 20), and (d) those with abnormal C3 conversion initially which was normalized during the study period (Patients 16, 17, 18, 19, 22). There did not appear to be any relationship between C3 conversion and septicemia, since the distribution of septic and non-septic patients within the groups was equal.

The ability of sera with reduced C3 conversion from septic and non-septic patients to reduce C3 conversion when added to PNHS was next determined. Ten µl, 20 µl, and 50 µl of burn sera were added to 50 µl of PNHS, the volumes were equalized with 0.15M saline, and C3 conversion by 10 mg/ml of inulin was measured. Controls for the experiments were as follows: (a) untreated PNHS added to PNHS, (b) factor B depleted normal human serum (RB) added to PNHS, and (c) factor D depleted normal human serum (RD) added to PNHS. For each patient, the temporal sequence of C3 conversion by inulin and C3 concentration are shown in the left figure (Figure A), and the inhibitory activity of multiple sera from the patients in comparison to the controls is shown in the right figure (Figure B). The numbers following the lines in the right figures indicate the postburn day serum which was tested in the assay. Figure 6 shows the data obtained on the non-septic burned patients. With the exception of serum obtained on day 24 from Patient 14, the C3 conversion values obtained when burn sera were added to PNHS were equal to or greater than the values obtained when the complement deficient control sera were added to PNHS. In addition, no correlation between C3 concentration and C3 conversion in the burn sera was demonstrated.

Identical results were obtained with the sera from the septic patients (Figure 7). Burn sera added to PNHS resulted in C3 conversion values equal to or greater than values obtained when the complement deficient control sera were added to PNHS. Also, no correlation between C3 concentration and C3 converting activity in the sera was demonstrated. These results indicated that reduction in C3 conversion in the burn sera was caused by a deficiency of critical normal serum proteins required for C3 conversion and not to an inhibitory activity.

To determine if reduction in C3 conversion in the burn sera was caused by a complement deficiency, three different sera from Patient 13 were supplemented with increasing concentrations of heated PNHS (56°C, 30 minutes) or untreated PNHS. At a concentration of 50%, untreated PNHS restored C3 conversion by inulin in the burn sera to normal (Figure 8). In contrast, heated PNHS (Δ PNHS)

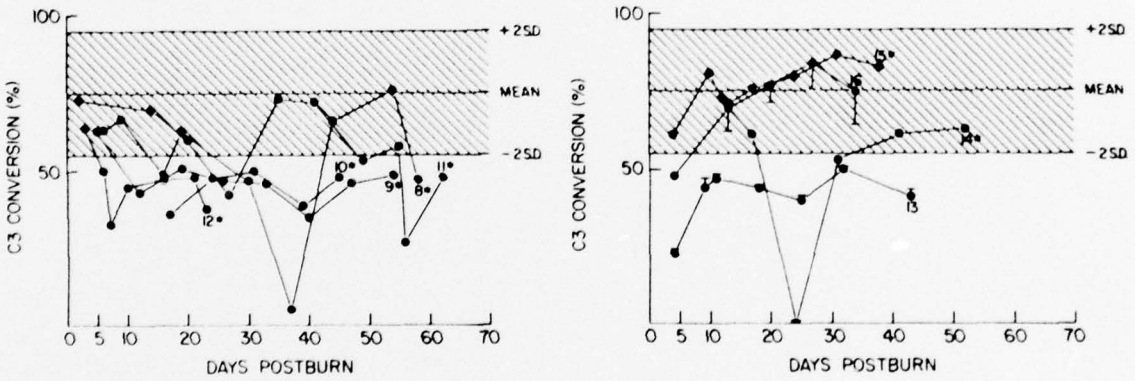


Figure 4. Conversion of C3 by inulin in sera obtained from nine non-septic burned patients during 62 days postburn. The shaded areas represent the range of C3 conversion for 20 normal individuals (mean $\pm$ 2 S.D.). The numbers following each line represent patient numbers. Refer to Table 1 for clinical characteristics of the patients. The points represent mean values of duplicate determinations, and each vertical bar represents the standard error of the mean. The asterisks following the patient numbers indicate that the values for C3 conversion in the sera were derived from single determinations.

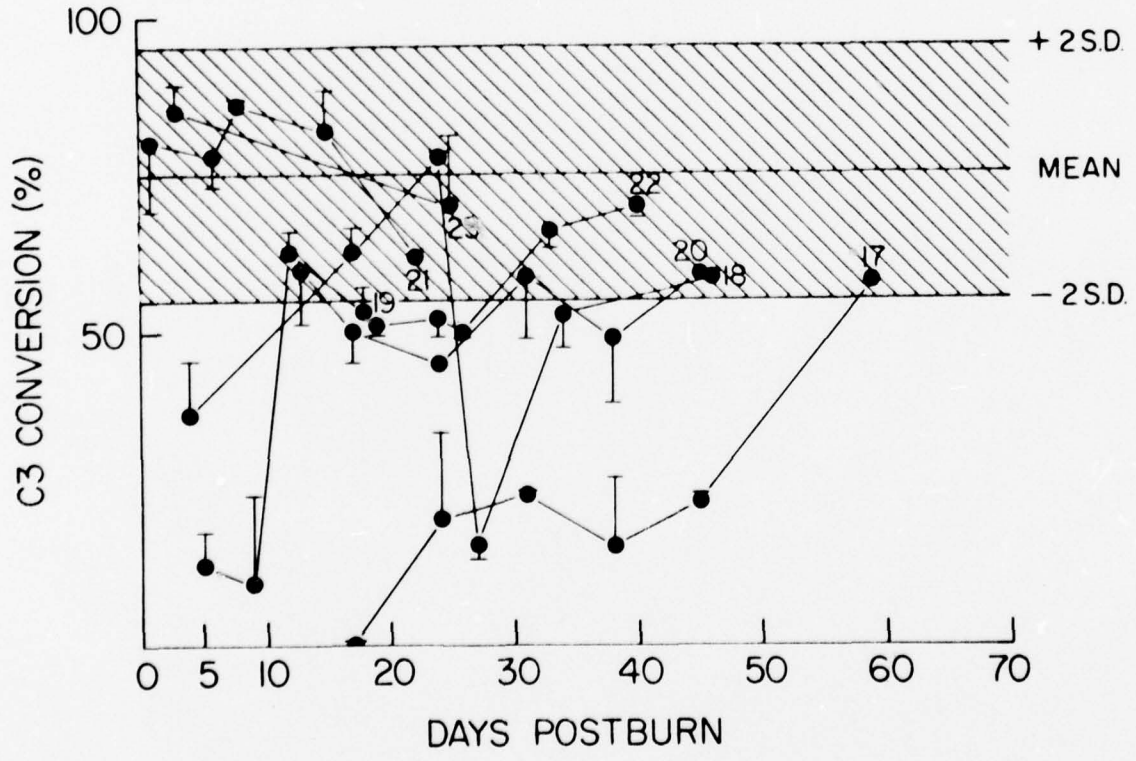


Figure 5. Conversion of C3 by inulin in sera obtained from seven septic burned patients during 58 days postburn. The shaded area represents the range of C3 conversion for 20 normal individuals (mean $\pm$ 2 S.D.). The numbers following each line represent patient numbers. Refer to Table 1 for clinical characteristics of the patients. The points represent mean values of duplicate determinations, and each vertical bar represents the standard error of the mean.

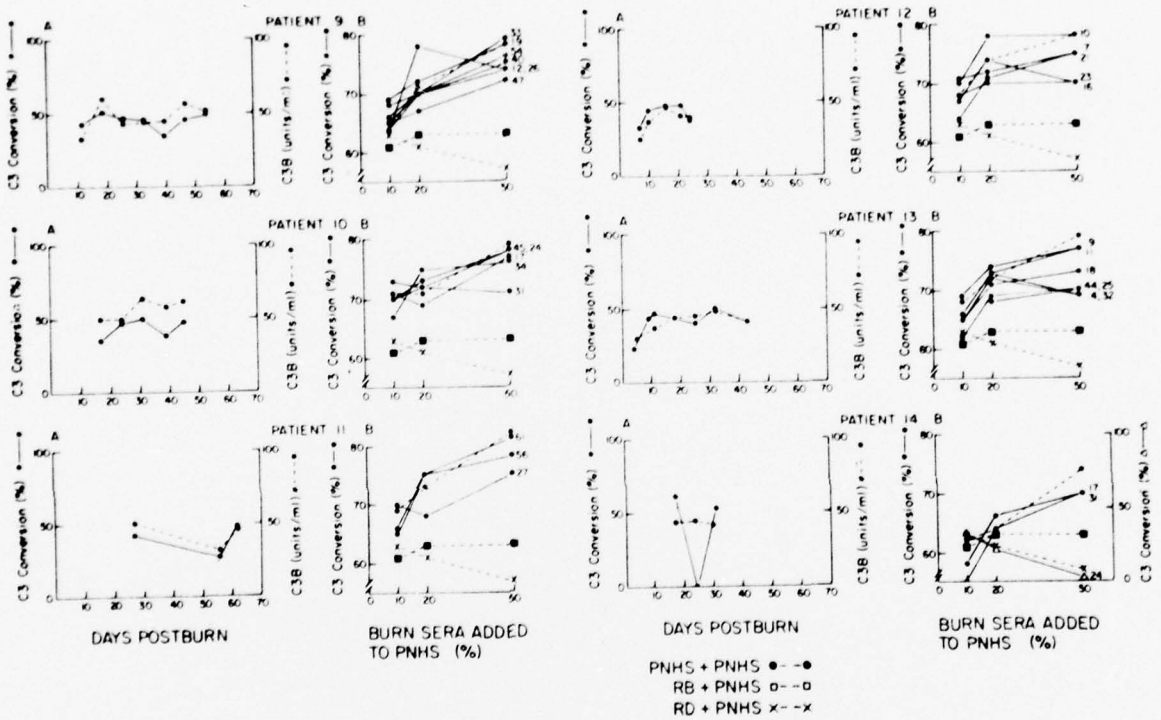


Figure 6. Temporal sequence of C3 conversion by inulin and C3 concentration in the sera of six non-septic burned patients, and the inhibitory activity of multiple sera from each patient. C3 conversion by inulin and B antigen of C3 concentration in the sera are presented in Figure A. C3 conversion values when increasing concentrations of burn sera were added to PNHS are shown in Figure B. The dotted lines in Figure B represent the controls: (a) untreated PNHS added to PNHS (•---•), (b) factor B depleted normal human serum (RB) added to PNHS (■---■), and (c) factor D depleted normal human serum (RD) added to PNHS (x---x). The numbers following the lines in this figure represent the postburn day serum that was tested in the assay. Serum obtained on day 24 from Patient 14 is referred to by the designation (Δ---Δ). The points represent mean values of three or more determinations.

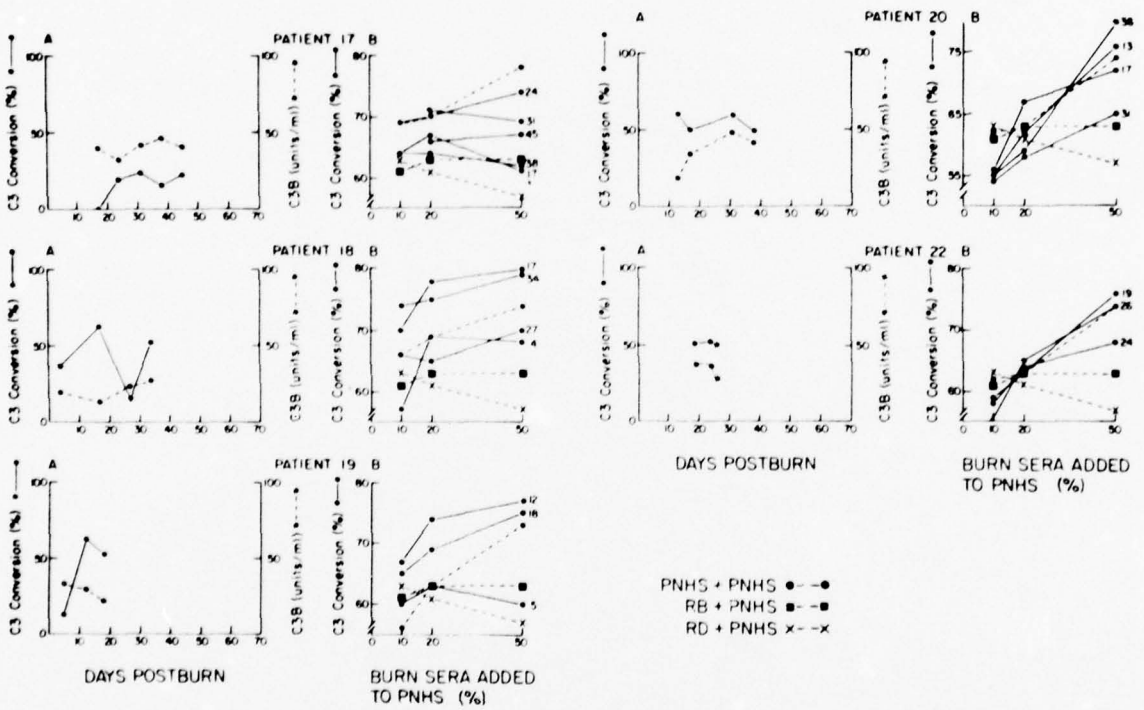


Figure 7. Temporal sequence of C3 conversion by inulin and C3 concentration in the sera of five -septic burned patients, and the inhibitory activity of multiple sera from each patient. C3 conversion by inulin and B antigen of C3 concentration in the sera are presented in Figure A. C3 conversion values when increasing concentrations of burn sera were added to PNHS are shown in Figure B. The dotted lines in Figure B represent the controls: (a) untreated PNHS added to PNHS (●---●), (b) factor B depleted normal human serum (RB) added to PNHS (■---■), and (c) factor D depleted normal human serum (RD) added to PNHS (x---x). The numbers following the lines in this figure represent the postburn day serum that was tested in the assay. The points represent mean values of three or more determinations.

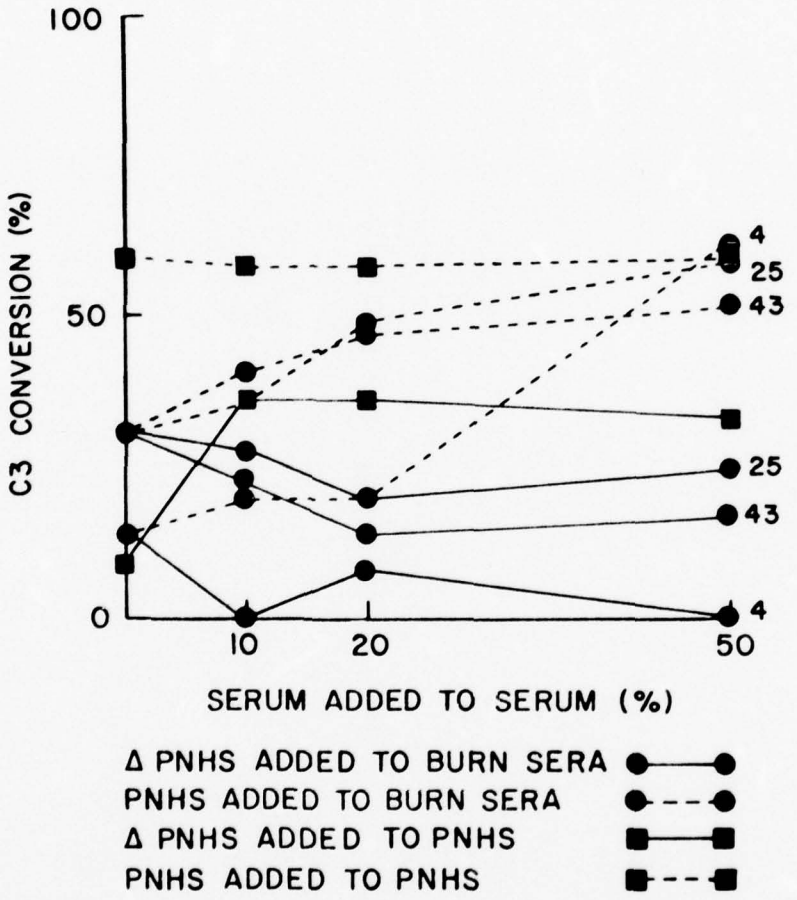


Figure 8. C3 conversion by inulin in sera from Patient 13 supplemented with untreated PNHS (●---●) or ΔPNHS (56°C, 30 minutes) (●——●). The numbers following the lines represent the postburn day serum that was tested in the assay. Untreated PNHS added to PNHS (■---■), and ΔPNHS (56°C, 30 minutes) added to PNHS (■——■) served as controls for the experiment. The points represent values of single determinations.

which lacked an intact complement system did not restore C3 conversion by inulin in the burn sera to normal or even to the levels obtained when Δ PNHS was added to untreated PNHS. These results indicated that reduction in C3 conversion in the burn sera was caused by a deficiency of one or more complement components.

Our next experiments were designed to provide preliminary evidence regarding the identity of the deficient complement protein or proteins causing reduced C3 conversion by inulin in the burn sera. Untreated PNHS was fractionated by molecular sieve chromatography on Sephadex G-200 or Sephadex G-75. Equal parts of excluded or included peaks and burn sera from Patient 13 were mixed together, and C3 conversion by 10 mg/ml of inulin was measured after 60 minutes of incubation at 37°C. The excluded and included peaks from Sephadex G-200 fractionation of PNHS restored C3 conversion in the burn sera to normal (Table 2). These results indicated that proteins greater and less than 200,000 daltons were deficient in the patient's sera causing reduced C3 conversion. Only the excluded fraction from the G-75 fractionation of PNHS restored C3 conversion in the burn sera to normal, indicating that proteins over 75,000 daltons were deficient in the patient's sera. Values obtained when the included peak from G-75 fractionation was added to the burn sera were greater than those obtained for unsupplemented burn sera. These results indicated that one or more proteins of less than 75,000 daltons were also contributing to reduction in C3 conversion in the burn sera.

In all of the experiments which we have performed to date, C3 conversion has only been evaluated utilizing reduction in the B antigenic determinant of C3 as the parameter for measuring C3 conversion, and inulin has been used as the only activating substance. Our next experiments were designed to further evaluate the integrity of the complement system in the burned patients using additional methods and activating substances. C3 and C5 levels and C3 and C5 conversion by inulin in burn sera treated with 10 mM ethylene glycol tetra-acetic acid (EGTA) and 10 mM $MgCl_2$ and in untreated burn sera were measured utilizing standard hemolytic methods. Treatment of the sera with EGTA and magnesium was used to block classical pathway activity. In addition, C3 conversion by cobra venom factor (CoVF) was measured in the burn sera utilizing the B antigen of C3 reduction assay.

Linear regression analyses were performed from determinations on multiple sera obtained from Patients 13 to 16. Immunochemical concentrations of C3 and C5 correlated well with concentrations of these proteins as measured by hemolytic methods (Figure 9). The data were converted from CH_{50} units/ml (hemolytic assays) and from mg% and units/ml (immunochemical assays) to percent of normal mean values to allow comparisons to be made between the assays. The results indicated that higher percentages were obtained with the hemolytic assays; the higher percentages did not however affect the correlations. Conversion of C3 by inulin in the burn sera utilizing the hemolytic and B antigen of C3 reduction assays also correlated well (Figure 10). Conversion of C3 by inulin in the burn sera also correlated with C3 conversion by another known activating substance of the alternative complement pathway, CoVF (Figure 11). Correlation between C3 and C5 conversion by inulin in the burn sera utilizing the hemolytic assays was also significant (Figure 12). Hemolytic C3 and C5 conversion by inulin in untreated burn sera correlated with C3 and C5 conversion by inulin in burn sera treated with EGTA and $MgCl_2$ (MgEGTA) (Figure 13). Values for C3 conversion in MgEGTA burn sera were equivalent to those in untreated sera, however C5 conversion values in MgEGTA

Table 2. Restoration of the C3 Converting Activity of Sera from Patient 13 with Fractions of PNHS Prepared by Molecular Sieve Chromatography

	C3 Conversion (%)[a]					
	No Fractionation		Sephadex G-200[b] Fractionation		Sephadex G-75[b] Fractionation	
Postburn[c] Day	Serum[d] Alone	Serum + PNHS	Serum + EP[e]	Serum + IP[f]	Serum + EP[e]	Serum + IP[f]
4	6	43	54	48	68	21
9	39	62	76	71	83	54
11	37	54	68	65	82	47
18	30	42	62	50	62	33
25	35	40	59	49	65	47
32	40	52	59	59	65	51
44	28	46	63	54	68	45
PNHS	64	64	76	75	71	44

[a]Values obtained from a single determination are represented.

[b]Fifty percent of the designated fraction was added to 50% of burn sera or PNHS, and C3 conversion by 10 mg/ml of inulin was measured.

[c]Postburn day referes to the postburn day serum tested in the assays.

[d]Percent of C3 conversion by inulin in unsupplemented burn sera.

[e]EP = excluded peak after fractionation of PNHS

[f]IP = included peak after fractionation of PNHS.

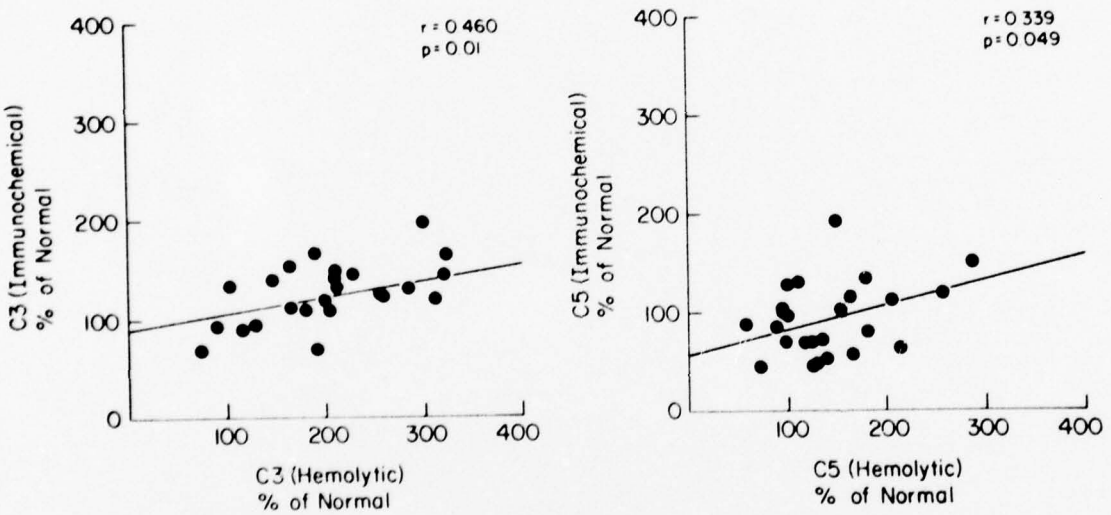
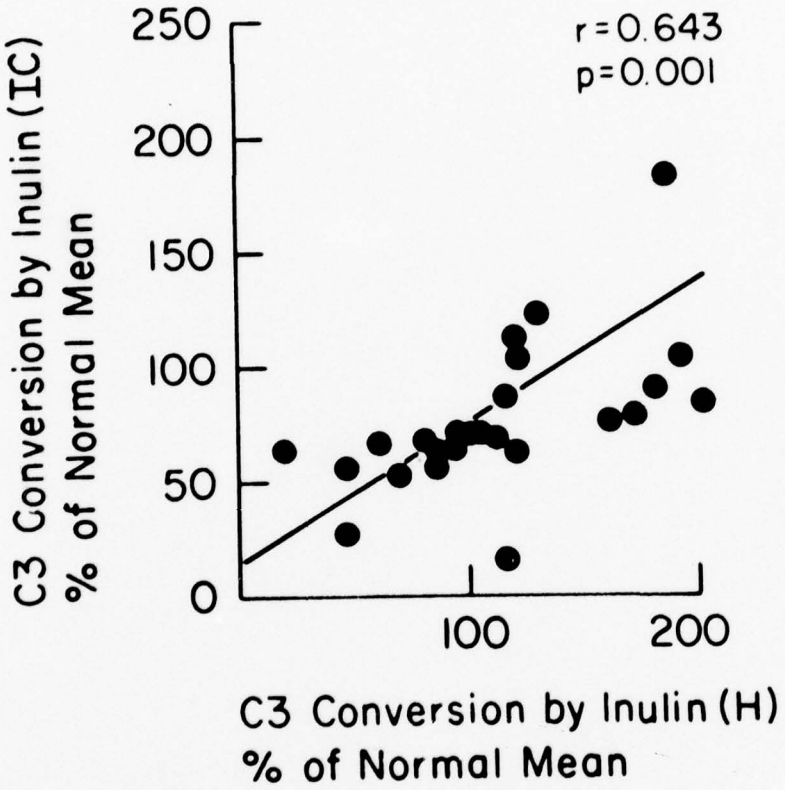
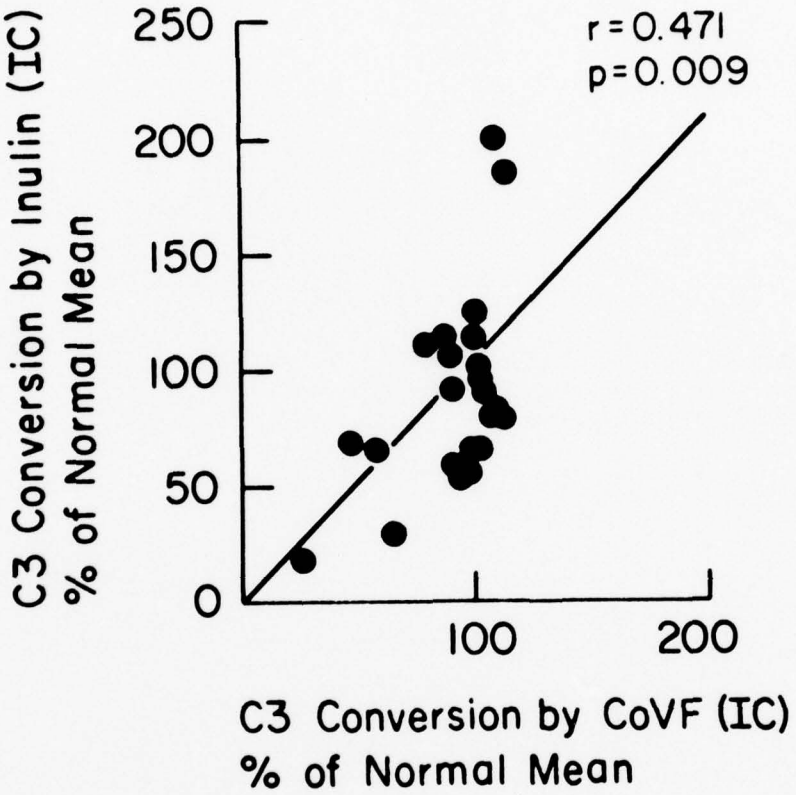


Figure 9. Relationship between concentrations of C3 and C5 in sera from Patients 13 to 16 measured by immunochemical and hemolytic methods. The coefficient of linear correlation (r) for the C3 determinations was 0.460, and the p value was 0.01. The r value for the C5 determinations was 0.339, and the p value was 0.049. The points represent mean values of duplicate determinations for each function.



IC = Immunochemical
H = Hemolytic

Figure 10. Relationship between C3 conversion by inulin in sera from Patients 13 to 16 utilizing the B antigen of C3 reduction and hemolytic assays. The coefficient of linear correlation (r) was 0.643, and the p value was 0.001. The points represent mean values of duplicate determinations for each function.



IC = Immunochemical

Figure 11. Relationship between C3 conversion by inulin and C3 conversion by CoVF in sera from Patients 13 to 16. Measurements using both activating substances were obtained utilizing the B antigen of C3 reduction assay. The coefficient of linear correlation (r) was 0.471, and the p value was 0.009. The points represent mean values of duplicate determinations for each function.

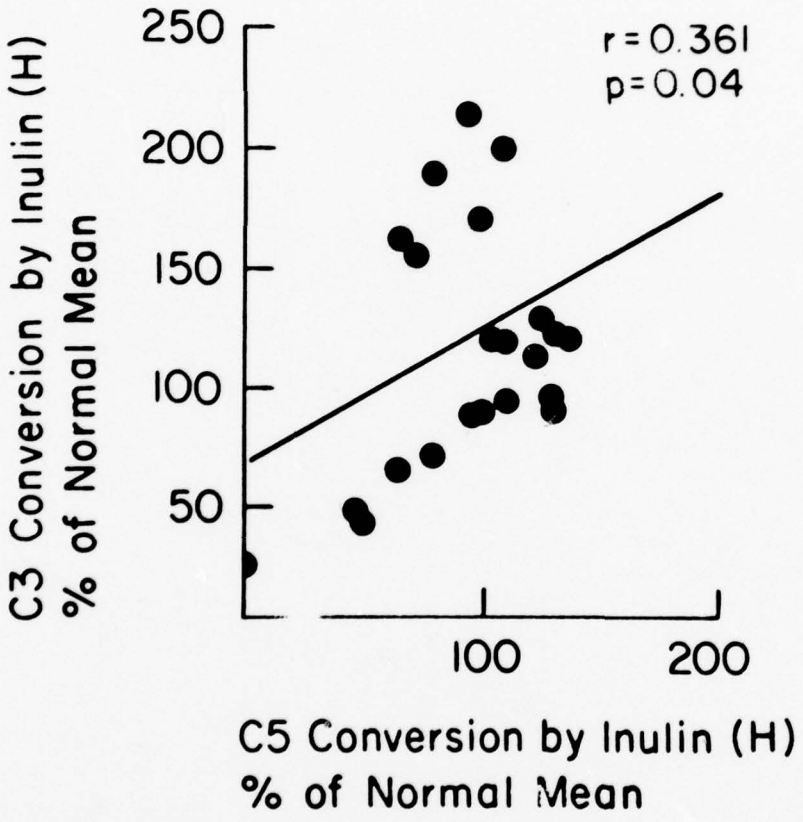


Figure 12. Relationship between C3 and C5 conversion by inulin in sera from patients 13 to 16. Hemolytic methods were used for all determinations. The coefficient of linear correlation (r) was 0.361, and the p value was 0.04. The points represent mean values of duplicate determinations for each function.

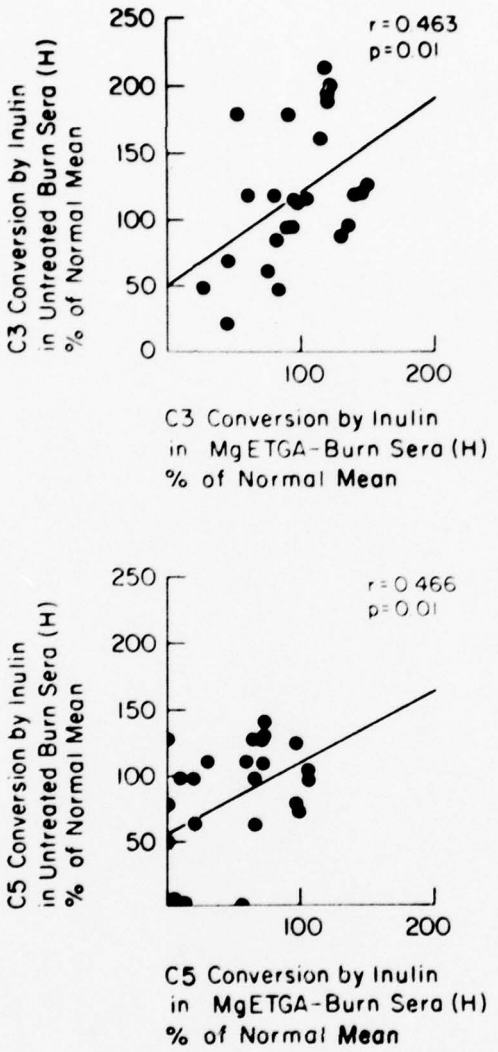


Figure 13. Relationship between C3 and C5 conversion by inulin in untreated sera from Patients 13 to 16 and in the same sera treated with EGTA and supplemented with $MgCl_2$ (MgEGTA). Hemolytic methods were used for all determinations. The coefficient of linear correlation (r) for C3 conversion in untreated and MgEGTA sera was 0.463, and the p value was 0.01. The coefficient of linear correlation (r) for C5 conversion in untreated and MgEGTA sera was 0.466, and the p value was 0.01. The points represent mean values of duplicate determinations for each function.

sera were lower than those in untreated burn sera. Since classical pathway activation is blocked in MgEGTA sera, the results indicated that C3 conversion by inulin measured alternative pathway activity, and that C5 conversion by inulin measured classical as well as alternative pathway activity.

To further examine the functional activities of factors B and D and the total alternative complement pathway, hemolytic radial immunodiffusion assays were set up according to previously published methods (62,63). Utilizing the same lots of agarose, guinea pig erythrocytes, and other reagents, a wide variability in results from experiment to experiment was obtained. Three different experiments on ten normal individual sera for the total alternative pathway assay are shown in Table 3. As shown in Table 4, purified human factor B restored hemolysis to normal in the factor B hemolytic assay, however the results on the normal sera among experiments were not reproducible. Purified human factor D did not restore hemolysis in the factor D hemolytic assay, and the reproducibility in the results among experiments was extremely poor (Table 5). For these reasons, the hemolytic radial immunodiffusion assays were not used to evaluate the alternative complement pathway in the burn sera.

b. Discussion

The observations reported in this study refuted the hypothesis that reduction in C3 conversion by inulin in the burn sera was caused by elevation of one or more regulatory proteins of the alternative complement pathway. C3b INA and β1H are regulatory proteins of the alternative pathway, and if present in elevated amounts in serum, could cause inhibition of C3 conversion. C3b INA inactivates C3b, thereby inhibiting the amplification loop of the alternative pathway formed by the C3 convertases C3b,B and C3b,B,P (64-67). β1H potentiates the inactivation of C3b by C3b INA and, in addition, directly inhibits C3b and the activity of the alternative pathway convertases C3b,B and C3b,B,P (22,23).

No correlation between reduction in C3 conversion by inulin and elevation of β1H and/or C3b INA in multiple burn sera was demonstrated. No significant differences were observed between the ability of burn sera with reduced C3 conversion and complement deficient normal sera to inhibit C3 conversion by inulin when added in increasing concentrations to PNHS. In addition, euglobulin fractions of burn sera were found to inhibit C3 conversion when added in increasing concentrations to PNHS, however the inhibitory activity of the euglobulin fractions was found to be unrelated to the original C3 converting activity of the burn sera. This observation also ruled out the possibility that burn injury altered a normal euglobulin protein to a configuration with regulatory activity, thereby inhibiting C3 conversion.

Several pieces of preliminary experimental evidence support the thesis that reduction in C3 conversion in the burn sera was caused by a deficiency of critical normal complement proteins required for C3 conversion. Restoration of C3 conversion by inulin in multiple burn sera was achieved by addition of PNHS. PNHS heated at 56°C for 30 minutes to destroy complement activity did not restore C3 conversion by inulin in the burn sera to normal. More than one complement protein caused decreased C3 conversion in the burn sera, one or more proteins with a molecular weight of greater than 200,000 daltons and one or more proteins with a molecular weight of less than 200,000 daltons. This conclusion is based on the experimental observation that restoration of C3 conversion by inulin in multiple

Table 3. Hemolytic Radial Immunodiffusion of the Total Alternative Pathway in Ten Normal Sera

Sera	Percent of Normal		
	Experiment 1	Experiment 2	Experiment 3
1[a]	98	160	106
2	98	68	106
3	98	90	132
4	87	120	106
5	50	68	106
6	98	90	132
7	152	90	132
8	152	160	132
9	126	200	82
10	152	120	106
PNHS[b]	126	120	82

[a]Numbers refer to the normal individuals from whom the sera were obtained.

[b]PNHS = pooled normal human serum.

Table 4. Hemolytic Radial Immunodiffusion of the Functional Activity of Factor B

Sera or Purified Protein	Percent of Normal		
	Experiment 1	Experiment 2	Experiment 3
1[a]	82	50	120
2	50	25	100
3	25	36	74
4	< 25	142	110
5	50	74	84
6	25	25	159
7	< 25	50	150
8	220	40	59
9	100	142	50
10	50	50	72
FB[b]	100	105	125
PNHS[c]	61	150	100

[a]Numbers refer to the normal individuals from whom the sera were obtained.

[b]FB = purified human factor B.

[c]PNHS = pooled normal human serum.

Table 5. Hemolytic Radial Immunodiffusion of the Functional Activity of Factor D

Sera or Purified Protein	Percent of Normal	
	Experiment 1	Experiment 2
1[a]	100	88
2	100	100
3	180	100
4	122	100
5	150	240
6	150	200
7	180	66
8	150	200
9	180	220
10	180	150
$\overline{FD}$[b]	0	0
PNHS[c]	150	97

[a]Numbers refer to the normal individuals from whom the sera were obtained.

[b]$\overline{FD}$ = purified factor $\overline{D}$.

[c]PNHS = pooled normal human serum.

burn sera was achieved by included and excluded peaks of PNHS after Sephadex G-200 or G-75 fractionation.

The assay for measuring C3 conversion utilizing reduction in the B antigenic determinant of C3 which has been used throughout our studies correlated well with standard hemolytic assays utilizing EAC$\overline{142}$ cells and purified classical complement components (68). Utilizing the B antigen reduction assay, results obtained when inulin was used as an activating substance correlated with results obtained when CoVF was used as the activating substance. Utilizing hemolytic assays, conversion of C3 by inulin correlated with conversion of C5 by inulin in the burn sera. The C3 conversion assays were shown to measure alternative complement pathway activity, and the C5 conversion assay measured classical as well as alternative pathway activity. These observations strengthen the concept that an abnormality of the alternative complement pathway is caused by burn injury which blocks the pathway prior to the C3 conversion step.

2. Studies to determine the association between septicemia and changes in complement and opsonins in burned patients

a. Results

In our previous preliminary studies, reduction in the immunochemical levels and functional activities of components of the classical and alternative complement pathways was associated with septicemia in two burned patients (Studies on a group of five additional septic burned patients showed that reduction in the classical complement pathway occurred prior to and during septic episodes in three of the patients (16). In the other two patients, decreased classical pathway activity was also demonstrated prior to the developement of septicemia, but not consistently during septic episodes. Reduction in the functional activity of the alternative complement pathway was not found to be associated with septicemia in any of the five burned patients in this study group.

Reduction in classical complement pathway activity did not reduce the opsonic capacity of the patients' sera for the specific microorganisms causing infection (9). In only one patient, consumption of components of the classical pathway occurring during septicemia decreased the opsonic capacity of the patient's sera for her own infecting microorganism, an isolate of Escherichia coli; sera from the same patient which could not opsonize E. coli opsonized her infecting strain of Staphylococcus aureus normally. The microorganisms which were isolated from the other septic burned patients and used to test the opsonic capacity of the patients' sera were also, with one exception, strains of Staphylococci. Since there is evidence that S. aureus can be opsonized by normal IgG in the absence of complement (17,18), the lack of demonstration of reduction in the opsonic capacity of the patients' sera for their infecting strains of Staphylococci might have been related to the lack of a requirement for complement for opsonization of the strains.

The objectives of the present investigation were as follows: (a) To determine if the classical complement pathway was consumed preferentially during septicemia in burned patients, or if alternative complement pathway consumption could occur as well; (b) to determine under what circumstances the classical pathway was not consumed during septicemia; (c) to determine if the classical pathway was always reduced during the initial postburn period in patients who later developed septicemia; (d) to determine if reduction in serum opsonic activity occurred for certain infecting bacteria and not for others, as was observed in our previously

studied group of septic patients; (e) to evaluate the effects of the administration of blood products on the observed changes in humoral factors in septic and non-septic burned patients and on the outcome of the infections in the septic patients; (f) to provide a detailed study of the functional integrity of the alternative pathway in septic and non-septic burned patients; and (g) to provide information about the immunochemical concentrations of classical pathway components in non-septic burned patients, since in our previous studies only functional activity of the classical pathway was measured in this patient population.

Nineteen patients with severe thermal injury were followed during 9 weeks postburn. Patients who appeared to be at the greatest possible risk of infection because of burn size or age or both were selected for the study. Serum samples were obtained from the patients as soon after the injury as possible and then at weekly intervals. In those patients who developed septicemia, serum samples were also obtained two additional times per week until blood cultures became negative. Blood cultures were drawn on all patients at least one time per week by our staff, and additional blood cultures were drawn at the discretion of the attending physicians. This procedure was adopted for the purpose of documenting negative as well as positive cultures obtained on the patients. Septicemia was documented by clinical findings and positive blood cultures. The clinical criteria used for the diagnosis of septicemia were (a) chills and fever, (b) tachycardia, (c) hypotension, and (d) disorientation.

Seven of the nineteen patients had positive blood cultures during their clinical course. Clinical signs of septicemia were documented in all of these patients, when blood cultures were positive. Clinical characteristics of this group of patients and information regarding blood cultures are given in Table 6. The other 12 patients had negative blood cultures. Clinical characteristics of this group of burned patients are shown in Table 7. Eight of the twelve non-septic patients (#5 to 12) died within 6 days postburn. Since only one serum sample was obtained on each of these patients, the data on these samples have not been included in this report. The deaths of the eight burned patients limited our population of non-septic patients. However this problem was not unexpected, since we had selected only high risk patients for our study. Data from the non-septic patients will be presented first, followed by the data on the septic patients, to allow appropriate comparisons between the two patient populations to be made.

Total hemolytic complement (CH_{50}) fell within the normal range in all of the non-septic burned patients for the duration of the study, despite decreased immunochemical levels of C1q and C2 in the sera of Patient 2 during 10 days postburn (Figure 14). The immunochemical level of C4 was found to be normal in all of the patients for the duration of the study. The immunochemical level of native C3 was also found to be normal in all of the patients for the entire study period (Figure 15). However, the immunochemical level of C5 fell below 2 standard deviations from the normal mean in Patients 1 and 2 during 30 days postburn. Functional activities of C3 and C5 were also measured in the sera of the four patients by standard hemolytic methods. The normal ranges for the assays were so broad that only 1 standard deviation from the normal mean could be represented in the figure. All of the patients had normal or elevated C3 and C5 as measured by these methods. These results indicated that the functional activity of C5 in the sera of Patients 1 and 2 was normal, despite decrease in the concentration of this protein during 30 days postburn. This concept is further supported by the observation that total hemolytic complement, a collective measurement of the functional

Table 6. Clinical Characteristics of the Septic Burned Patients

Patient No.	Age	Sex[a]	Body Surface Injured[b] Total %	Third Degree %	Infecting Microorganisms	Blood Cultures[c] Positive Day	Negative Day
I	4	F	48	48	S. aureus	17,24,28,31,35, 36,37,38,40,42	18,24,45,52
II	3	F	77	70	E. coli S. aureus P. aeruginosa	10 17,18,20 23,29	4,30,41
III[d]	55	F	52	17	P. aeruginosa	17,18,19,20	5,9,12
IV	11	M	51	13	S. aureus	13,31,34	17,24,38,45
V[e]	14	F	38	35	S. aureus	37,39	19,23,25,32
VI[f]	9	F	67	67	S. aureus E. coli K. pneumoniae C. freundii S. faecalis P. aeruginosa	2 4 4,7,11,14,18,21,23 4 4,5,7,9 4	1
VII	37	M	59	54	S. aureus S. faecalis	15,24 15	5,13,17,20, 21,29

[a]M = male; F = female.

[b]All patients had flame burn injuries.

[c]Numbers indicate the number of days following the injury that positive or negative blood cultures were obtained.

[d]This patient died of septic shock on day 21.

[e]This patient died of septic shock on day 41.

[f]This patient died of septic shock on day 23.

Table 7. Clinical Characteristics of the Non-Septic Burned Patients

Patient No.	Age	Sex[a]	Body Surface Injured[b]	
			Total %	Third Degree %
1	59	M	62	0
2	14	M	43	33
3	12	M	40	0
4	15	M	49	45
5[c]	19	M	80	74
6[c]	49	M	60	31
7[c]	16 mos	M	69	69
8[c]	32	M	98	30
9[c]	13	F	47	35
10[c]	9	F	55	55
11[c]	56	F	48	16
12[c]	87	F	60	29

[a]M = male; F = female.

[b]All patients had flame burn injuries.

[c]These patients expired within 6 days postburn.

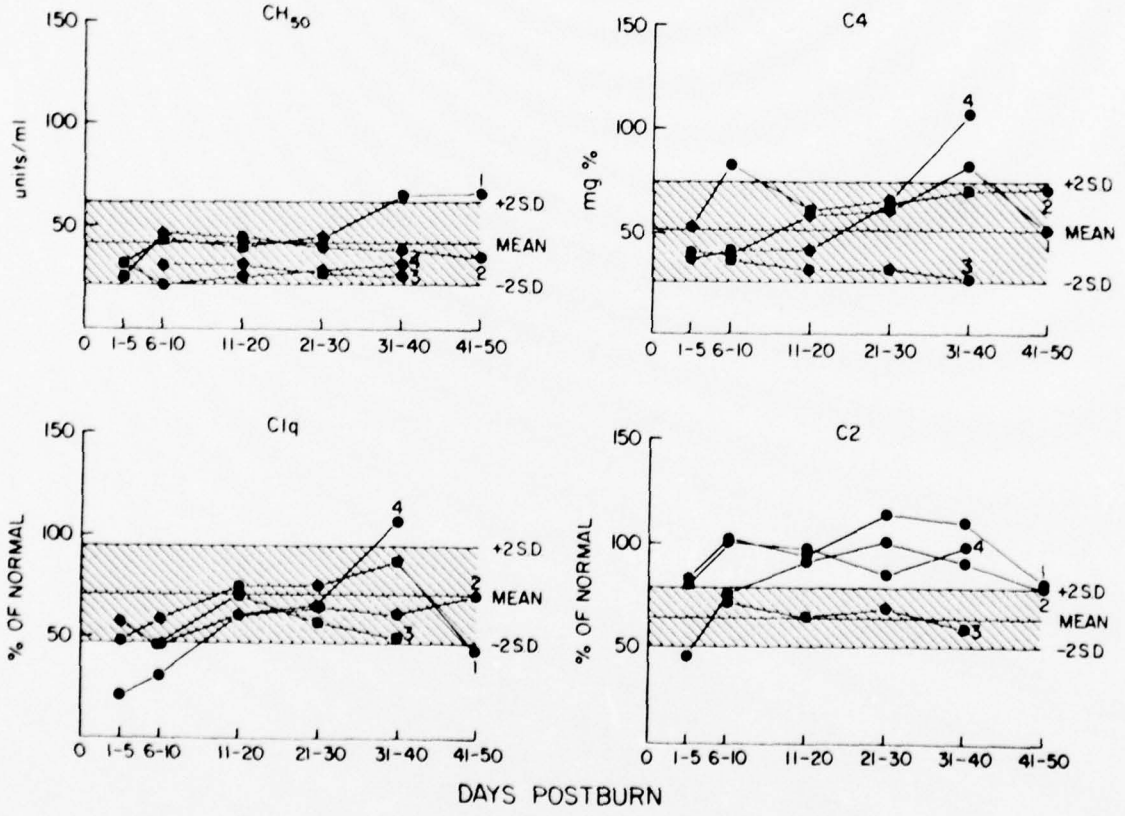


Figure 14. Immunochemical levels and functional activities of components of the classical complement pathway in the sera of four non-septic burned patients during 50 days postburn. The shaded areas represent the variation in 20 individual normal sera (mean $\pm$ 2 S.D.). The Arabic numerals following the lines represent patient numbers. Refer to Table 7 for the clinical characteristics of the patients. The points represent mean values of duplicate determinations.

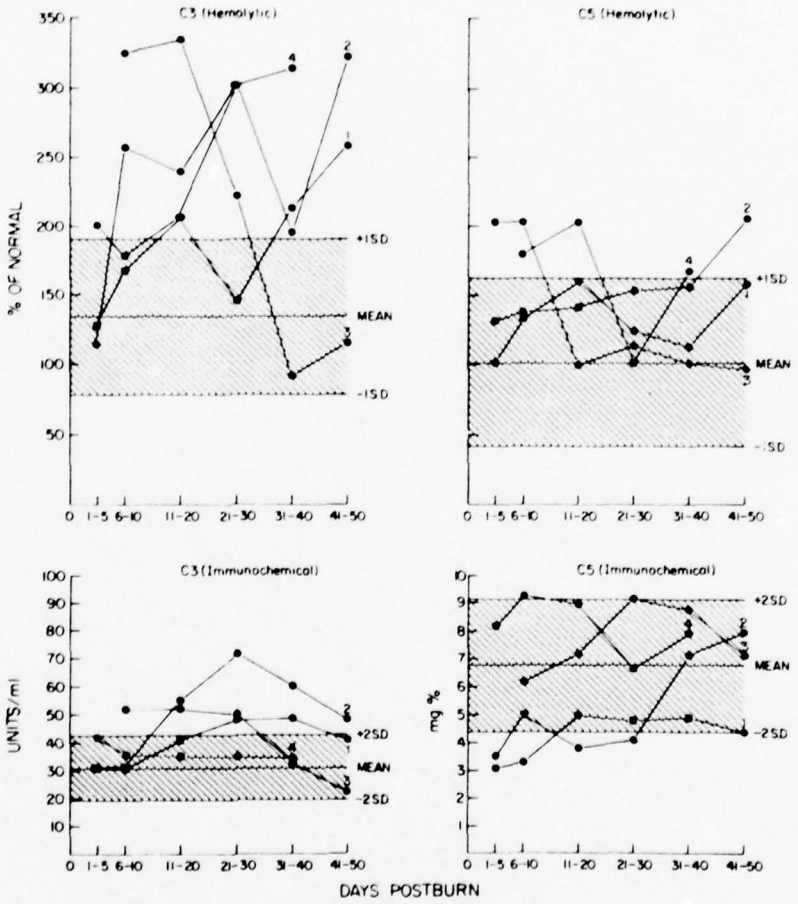


Figure 15. Immunochemical and hemolytic measurements of C3 and C5 in the sera of four non-septic burned patients during 50 days postburn. The shaded areas represent the variation in 20 individual normal sera (mean $\pm$ 1 S.D. for hemolytic determinations and mean $\pm$ 2 S.D. for immunochemical determinations). The Arabic numerals following the lines represent patient numbers. Refer to Table 7 for the clinical characteristics of the patients. The points represent mean values of duplicate determinations.

activities of C1 to C9, was normal in the sera of Patients 1 and 2 despite decreased immunochemical levels of C5 and/or C1q and C2.

Properdin in one of the four patients was decreased below 2 standard deviations from the normal mean during 20 days postburn, and in the other three patients, fell below the normal mean value until the 41st to 50th postburn day period (Figure 16). C3b INA and factor B levels in all of the patients were normal or elevated during the entire study period.

Utilizing reduction in the B antigenic determinant of C3 by radial immunodiffusion as a method for measuring C3 conversion, Patient 1 had reduced C3 conversion by inulin during 5 days postburn and by cobra venom factor (CoVF) during 10 days postburn; C3 conversion by both activating substances in this patient's sera was restored to normal thereafter (Figure 17). Patient 2 had normal C3 conversion by inulin and CoVF until the 21st to 30th postburn day period which was subsequently restored to normal. Patients 3 and 4 had normal C3 conversion by both activating substances for the duration of the study utilizing this method. The hemolytic assay for measuring C3 conversion by inulin yielded different results, although the trends were the same. C3 conversion by inulin was reduced in Patient 1 during 30 days postburn and was subsequently normalized. C3 conversion was decreased during the 21st to 30th postburn day period in Patient 2, although it remained within the normal range. C3 conversion by inulin in Patients 3 and 4 was consistently normal for the duration of the study utilizing this method. Treatment of the burn sera with 10 mM ethylene glycol tetra-acetic acid (EGTA) and 10 mM $MgCl_2$, which blocked classical pathway activity, did not substantially reduce C3 conversion by inulin in the sera, and actually improved C3 converting activity during the first 10 days postburn in Patient 1. C5 conversion was normal for the entire study period except in Patient 1 during the 31st to 40th postburn day period and in Patients 2 and 3 during the 21st to 30th postburn day period. C5 conversion by inulin in EGTA-treated magnesium supplemented sera (MgEGTA-burn sera) was markedly reduced in Patient 2 during the 21st to 30th postburn day period, in Patient 3 until the 31st to 40th postburn day period, and in Patient 1 for the duration of the study. C5 conversion in MgEGTA-burn sera was slightly reduced but fell within the normal range in Patient 4. From these results the following conclusions were drawn: (a) Utilizing two different activating substances and two different methods for measuring C3 conversion, C3 conversion was abnormal in two of the four study patients; (b) C5 conversion by inulin was frequently abnormal when C3 conversion by inulin was normal and vice versa, and in only one patient did C3 and C5 conversion correlate exactly; (c) C3 conversion by inulin measured alternative pathway activity; (d) C5 conversion by inulin measured classical as well as alternative pathway activity; and (e) a wide variation in the results of duplicate determinations was observed using the hemolytic methods for measuring C3 and C5 conversion, in comparison to the method for measuring C3 conversion utilizing reduction in the B antigenic determinant of C3 by radial immunodiffusion.

Levels and activities of classical pathway components in the sera of the seven septic patients are shown in Figure 18. In all of the patients, CH_{50} was markedly reduced initially below the lowest value obtained in the non-septic patients (67% of the normal mean value). Patient VI was the only patient in whom classical pathway components C1q, C4, C2, C3 and C5, as well as CH_{50}, were reduced for the entire study period of 21 days. This patient also had the most severe bacterial infections with multiple microorganisms isolated from all blood cultures which

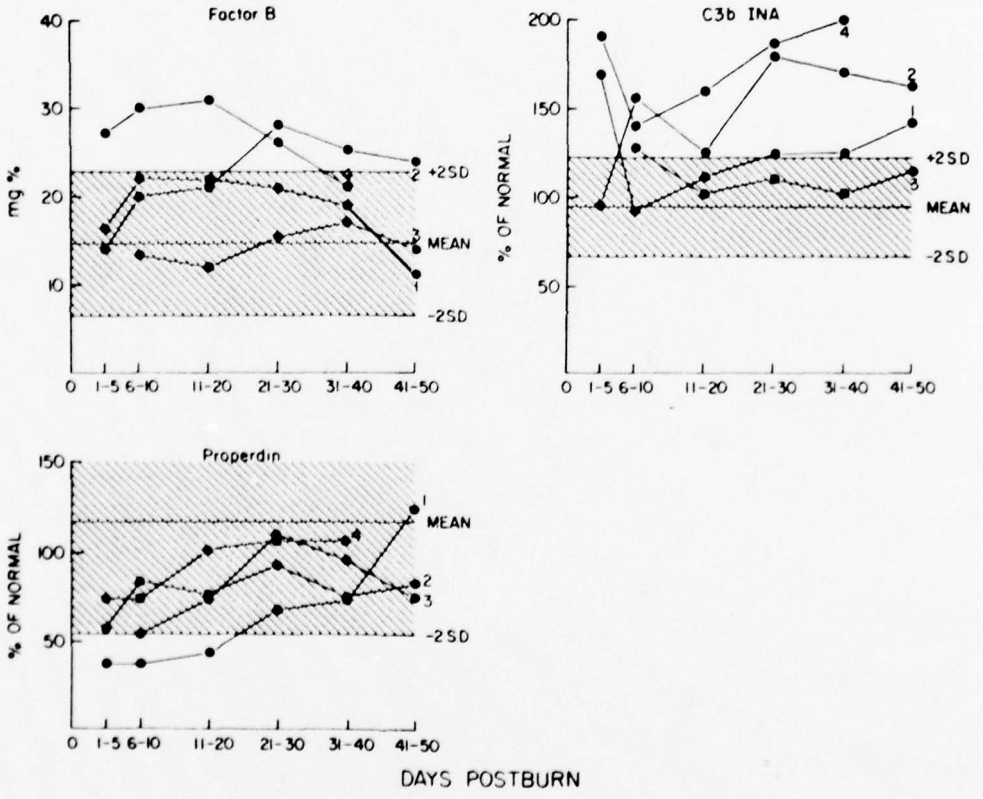


Figure 16. Immunochemical levels of factor B, properdin, and C3b inactivator (C3b INA) in the sera of four non-septic burned patients during 50 days postburn. The shaded areas represent the variation in 20 individual normal sera (mean $\pm$ 2 S.D.). The Arabic numerals following the lines represent patient numbers. Refer to Table 7 for the clinical characteristics of the patients. The points represent mean values of duplicate determinations.

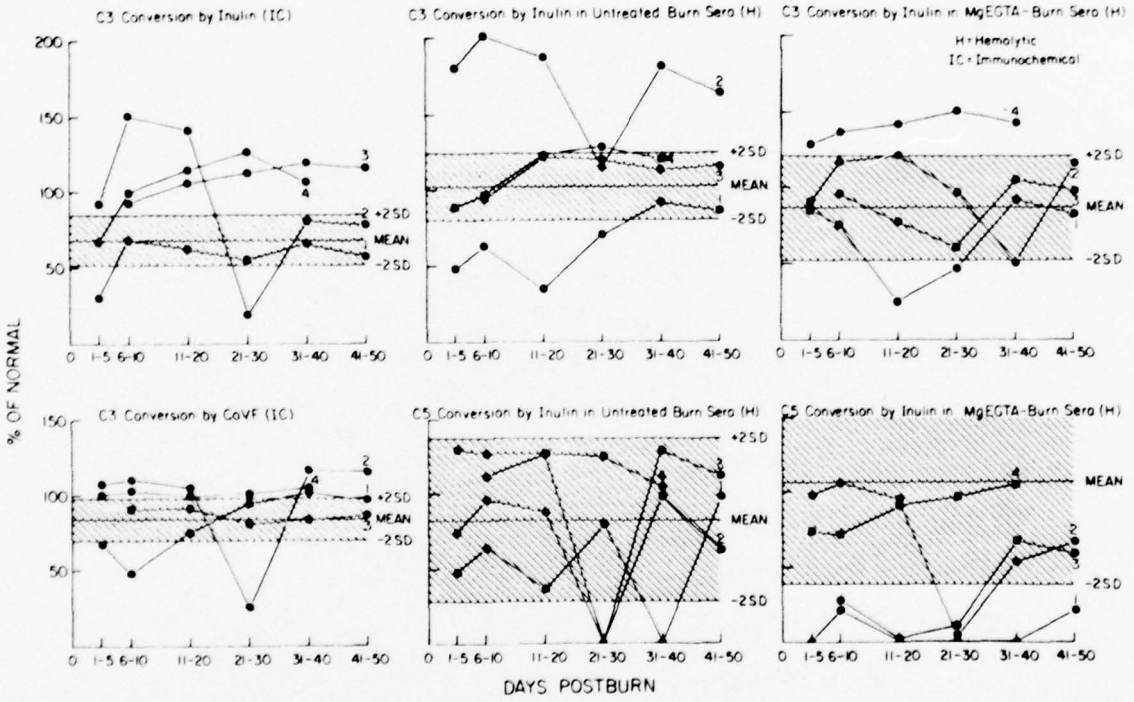
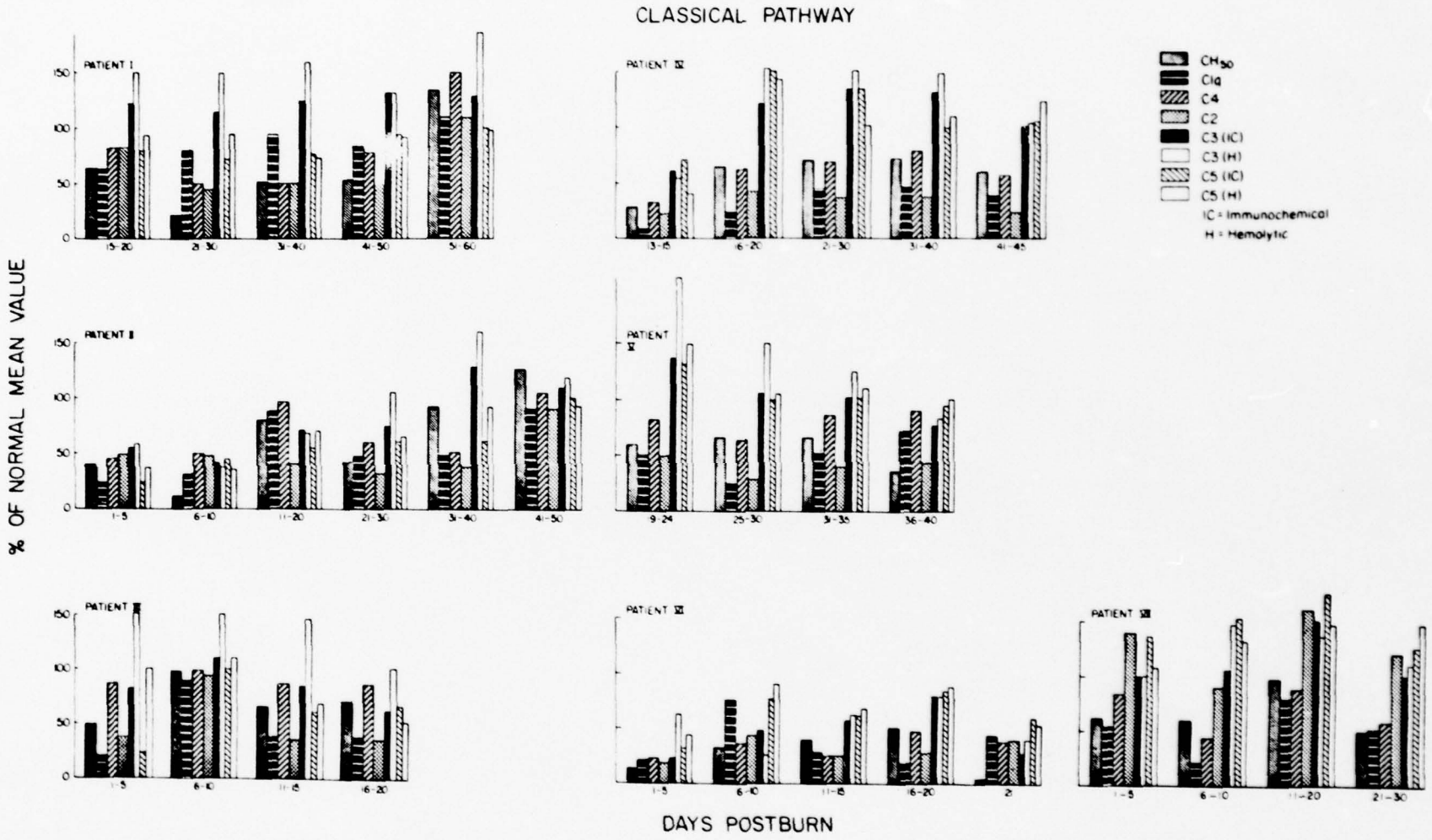


Figure 17. Functional activity of the alternative complement pathway in the sera of four non-septic burned patients during 50 days postburn. Conversion of C3 and C5 by inulin in untreated burn sera and in burn sera treated with 10 mM ethylene glycol tetra-acetic acid (EGTA) and 10 mM $MgCl_2$ (MgEGTA - burn sera) was measured by hemolytic methods. Conversion of C3 by inulin and cobra venom factor (CoVF) in untreated burn sera was also measured by reduction in the B antigenic determinant of C3 by radial immunodiffusion. The shaded areas represent the variation in 20 individual normal sera (mean $\pm$ 2 S.D.). The Arabic numerals following the lines represent patient numbers. Refer to Table 7 for the clinical characteristics of the patients. The points represent mean values of duplicate determinations.

Figure 18. Immunochemical and functional activities of the classical complement pathway in the sera of seven septic burned patients during 50 days postburn. The levels of C1q, C4, and C2 were determined immunochemically. C3 and C5 were measured by immunochemical and hemolytic methods. The Roman numerals represent the patient numbers. Refer to Table 6 for the clinical characteristics of the patients. The values of duplicate determinations for each function are presented. (Refer to page 49a for the figure.)


CLASSICAL PATHWAY
% OF NORMAL MEAN VALUE
DAYS POSTBURN
PATIENT I
PATIENT II
PATIENT III
PATIENT IV
PATIENT V
PATIENT VI
PATIENT VII
CH50
C1q
C4
C2
C3 (IC)
C3 (H)
C5 (IC)
C5 (H)
IC = Immunochemical
H = Hemolytic

were obtained after the first day postburn. Patient III also had consumption of C1q, C4, C2, C3, C5 and CH_{50} during the 20-day postburn period during which she was studied, except during the 6th to 10th postburn day period; the first positive blood culture was not obtained on this patient until day 17. Patient II had multiple episodes of septicemia during 10 to 29 postburn days, associated with decrease in all of the classical pathway components during this time. During the 31st to 40th postburn day period, the classical complement components except C3 were decreased in this patient and during the 41st to 50th postburn day period, all of the components were restored to normal values. Patient I, whose septic episodes occurred during days 17 to 42, had decreased CH_{50}, C1q, C4, C2, and C5, but not C3, through the 41st to 50th postburn day period, which were normalized thereafter. In two of the other patients (#IV and V), consumption of CH_{50}, C1q, C4, C2, but not C3 or C5, occurred throughout the study except initially (days 13 to 15) in Patient IV, and during the 36th to 40th postburn day period in Patient V. During these times, all of the components, including C3 and C5, were decreased. Patient IV had septic episodes on day 13 and days 31 to 34, and Patient V became septic on day 37. The last patient (#VII) had intermittent septic episodes during 15 to 24 days postburn; decrease in CH_{50}, C1q, and C4, but not C2, C3, or C5, occurred during the entire study period of 30 days postburn.

Decrease in C3b INA was demonstrated in four of the seven patients (Figure 19). C3b INA was decreased during the initial postburn period in three of the patients (#II, III, and IV) and for the duration of the study in Patient VI. C3b INA was also reduced in Patient III after the 6th to 10th postburn day period. Factor B was decreased concurrently with C3b INA except in Patient III. In Patient III, factor B was normalized after the first 5 days postburn and remained normal thereafter. Properdin was markedly reduced in all patients for the duration of the study.

C3 conversion by inulin and CoVF, and C5 conversion by inulin were reduced for the entire study period in Patient III, and until the 36th to 40th postburn day period in Patient V (Figure 20). C3 and C5 conversion was normal in Patient VI until day 21, probably because there were only small amounts of C3 and C5 in this patient's sera to be converted by large amounts of activating substances. In the other patients, C3 conversion was often reduced when C5 conversion was not and vice versa. In Patient I, C3 conversion was reduced for the entire study period, but C5 conversion was normalized during the 41st to 50th postburn day period. In Patient II, C3 conversion was reduced during the entire study period except during the 11th to 20th postburn day period. C5 conversion in this patient was only reduced during the initial 5 days postburn. In Patient IV, C5 conversion was reduced for the entire study period of 50 days, but C3 conversion was only reduced during 20 days postburn. In Patient VII, C3 conversion was reduced for the entire study period; however, C5 conversion was normal. In all of the patients, C3 and C5 conversion by inulin in the burn sera was either not reduced or was only slightly reduced by treatment of the sera with EGTA and $MgCl_2$. These results indicated that the C3 and C5 conversion methods measured alternative pathway activity in the burn sera.

Reduction in complement levels and activities did not decrease the opsonic capacity of the patients' sera for their own infecting microorganisms (Figure

Figure 19. Immunochemical levels of factor B, properdin, and C3b inactivator (C3b INA) in the sera of seven septic burned patients during 50 days postburn. The Roman numerals represent the patient numbers. Refer to Table 6 for the clinical characteristics of the patients. The values of duplicate determinations for each function are presented. (Refer to page 51a for the figure.)

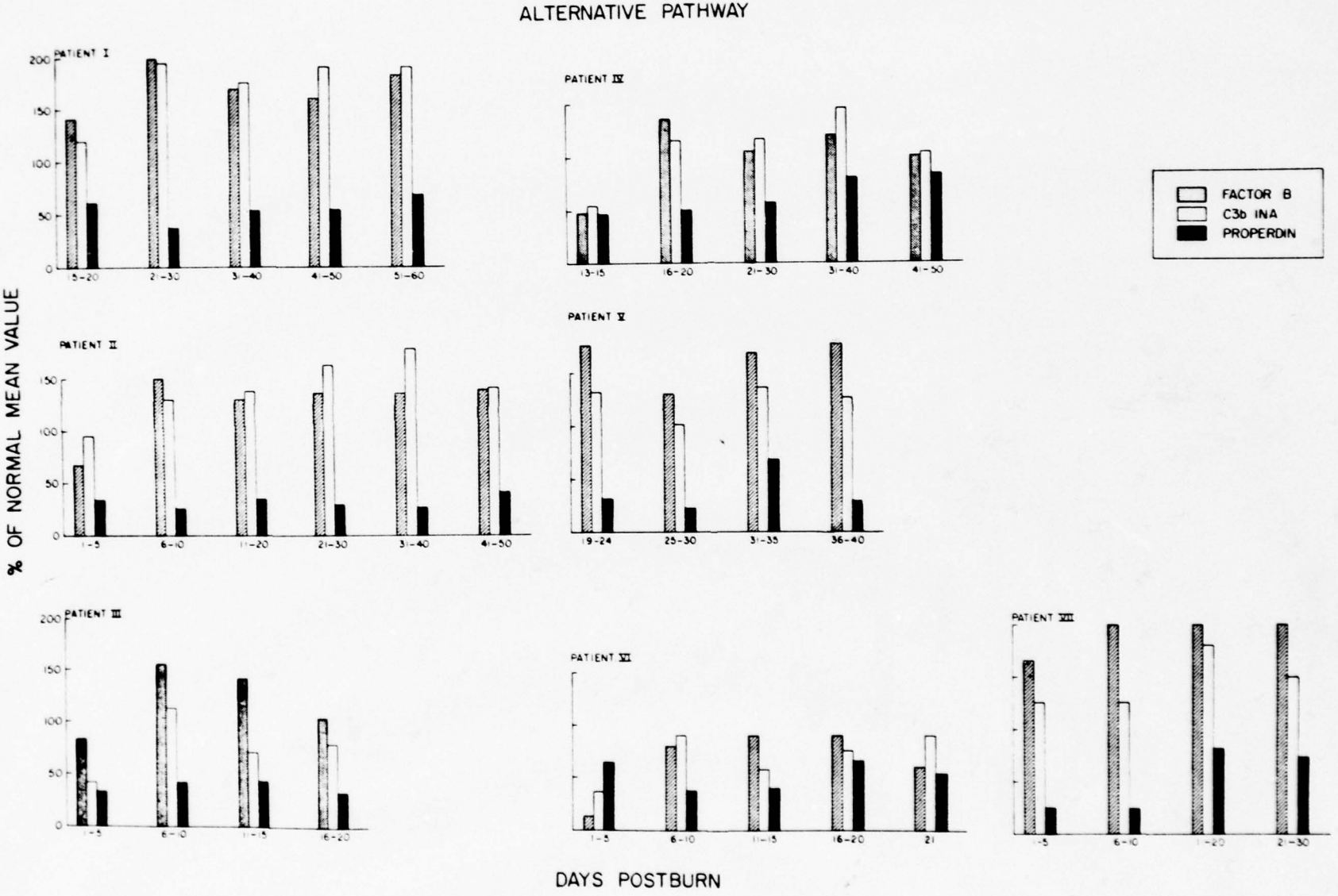
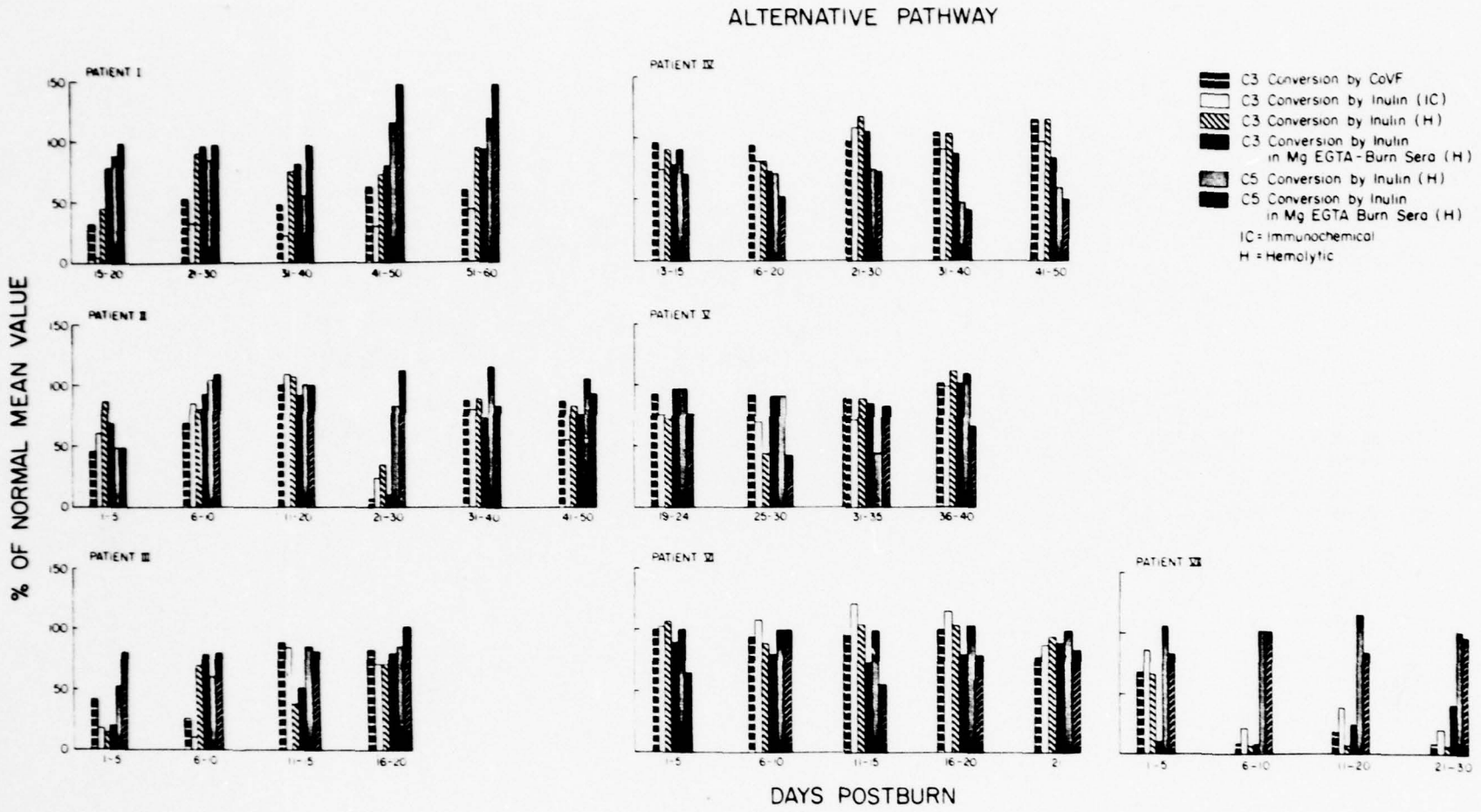

ALTERNATIVE PATHWAY
PATIENT I
PATIENT II
PATIENT III
PATIENT IV
PATIENT V
PATIENT VI
PATIENT VII
FACTOR B
C3b INA
Pproperdin
% OF NORMAL MEAN VALUE
DAYS POSTBURN

Figure 20. Functional activity of the alternative complement pathway in the sera of seven septic burned patients during 50 days postburn. Conversion of C3 and C5 by inulin in untreated burn sera and in burn sera treated with 10 mM ethylene glycol tetra-acetic acid (EGTA) and 10 mM $MgCl_2$ (MgEGTA-burn sera) was measured by hemolytic methods. Conversion of C3 by inulin and cobra venom factor (CoVF) was also measured by reduction in the B antigenic determinant of C3 by radial immunodiffusion. The Roman numerals represent the patient numbers. Refer to Table 6 for the clinical characteristics of the patients. The values of duplicate determinations for each function are presented. (Refer to page 52a for the figure.)


ALTERNATIVE PATHWAY
% OF NORMAL MEAN VALUE
DAYS POSTBURN
PATIENT I
PATIENT II
PATIENT III
PATIENT IV
PATIENT V
PATIENT VI
PATIENT VII
C3 Conversion by CoVF
C3 Conversion by Inulin (IC)
C3 Conversion by Inulin (H)
C3 Conversion by Inulin in Mg EGTA-Burn Sera (H)
C5 Conversion by Inulin (H)
C5 Conversion by Inulin in Mg EGTA Burn Sera (H)
IC = Immunochemical
H = Hemolytic

21). Although S. aureus was the most common infecting microorganism, E. coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Citrobacter freundii, and Streptococcus faecalis were isolated as well. In only one patient (#VI), opsonic activity of the patient's sera for her infecting strain of K. pneumoniae was reduced; the reduction occurred only during the first 5 days postburn.

The types and amounts of blood products administered to the septic patients are shown in Figure 22. A wide variation in the regimen for administration of blood products was observed. All of the patients received whole blood with Patients I and II receiving a total of less than 1000 ml, Patients III, V, and VI receiving a total of from 4000 to 6750 ml, and Patients IV and VII receiving a total of 9000 ml. Only four of seven septic patients received single donor plasma. Patient III received the least amount (approximately 2000 ml), and Patients IV, V, and VI received from 7200 ml to 8500 ml.

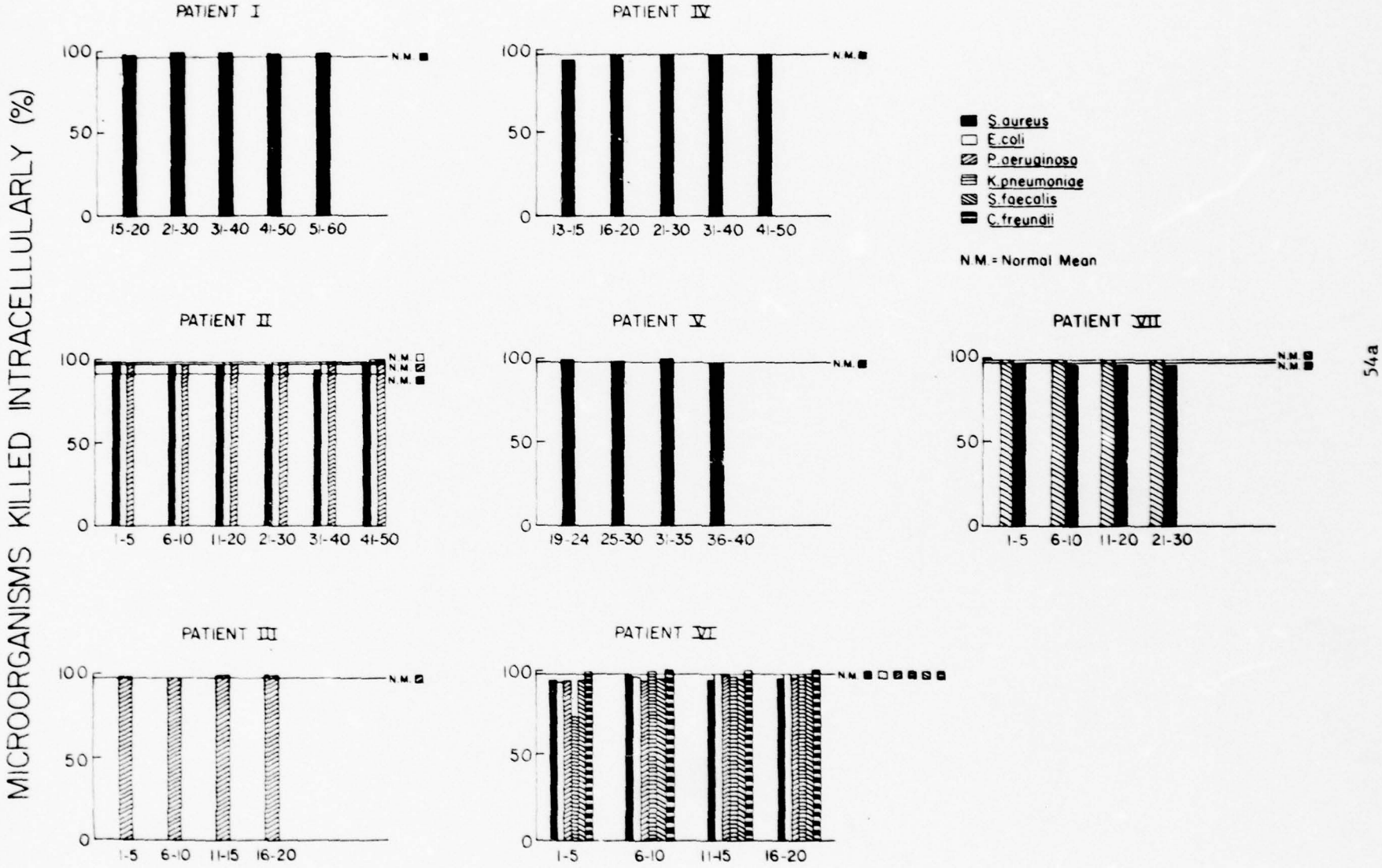
All of the non-septic burned patients received single donor plasma, and three of the four patients received whole blood during the study period (Figure 23). Patients 1, 3, and 4 received from 1250 ml to 2950 ml of single donor plasma during the study; Patient 2 received the most plasma, totaling approximately 8950 ml. This patient also received the most whole blood (12,485 ml), with Patients 3 and 4 receiving 795 ml and 4900 ml respectively. Per unit of time, all of the non-septic patients with the exception of Patient 2 received less whole blood and plasma than the four septic patients who received both blood products.

b. Discussion

The data presented in this section support our previous preliminary observations that consumption of the classical complement pathway was associated with and was caused by septicemia in thermally injured patients (9,16). In the previous study, only three of five septic burned patients had classical pathway consumption associated consistently with septic episodes. In this investigation, all of the seven study patients had decreased classical pathway activity during their septic episodes, and there were no exceptions. All of the patients who did not survive septicemia and who died of septic shock had consumption of all of the classical complement components (C1 to C5) during their septic episodes; the levels of C6 to C9 were not measured individually in our study. Patients who survived septicemia had multiple patterns of classical complement pathway consumption during their septic episodes as follows. (a) Consumption of all components, (b) consumption of only C1 and C4, (c) consumption of only C1, C4, and C2, and (d) consumption of C1, C4, C2, and C5, but not C3. In these patients, classical pathway activity was restored to normal within 20 days following the last positive blood culture. This observation is preliminary, however, since only two of the four surviving patients were studied long enough following septicemia to make this evaluation.

The alternative complement pathway was consumed in only one of the seven septic patients. Blood cultures were positive in this patient after the first postburn day every time they were obtained, and this patient died of septic shock on the 23rd postburn day. C3b INA and factor B were markedly decreased for the entire study period in this patient. Factor B was also decreased in three other

Figure 21. Serum opsonizing activity for the microorganisms causing septicemia in the seven burned patients during 50 days postburn. None of the microorganisms isolated from the patients and used in the opsonic assays was susceptible to killing by either leukocytes or serum alone. Serum concentrations in the assays varied for each microorganism and were based on the minimal concentration of pooled normal human serum which promoted maximal intracellular killing of the microorganism during the shortest incubation period. The Roman numerals represent the patient numbers. Refer to Table 6 for the clinical characteristics of the patients. The values of duplicate determinations for each function are presented. (Refer to page 54a for the figure.)


MICROORGANISMS KILLED INTRACELLULARLY (%)
PATIENT I
100
50
0
15-20 21-30 31-40 41-50 51-60
N.M.
PATIENT IV
100
50
0
13-15 16-20 21-30 31-40 41-50
N.M.
S.aureus
E.coli
P.aeruginosa
K.pneumoniae
S.faecalis
C.freundii
N.M. = Normal Mean
PATIENT II
100
50
0
1-5 6-10 11-20 21-30 31-40 41-50
N.M.
PATIENT V
100
50
0
19-24 25-30 31-35 36-40
N.M.
PATIENT VII
100
50
0
1-5 6-10 11-20 21-30
N.M.
PATIENT III
100
50
0
1-5 6-10 11-15 16-20
N.M.
PATIENT VI
100
50
0
1-5 6-10 11-15 16-20
N.M.

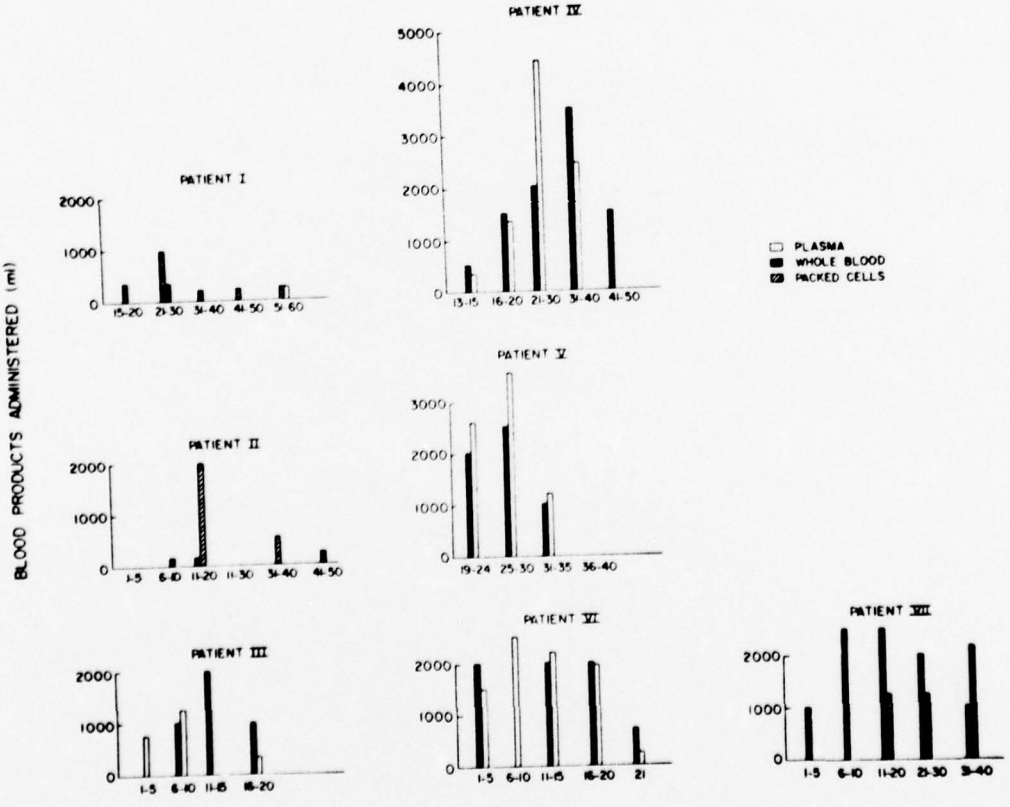


Figure 22. Temporal sequence of the administration of blood products to the seven septic burned patients. The Roman numerals represent the patient numbers. Refer to Table 6 for the clinical characteristics of the patients.

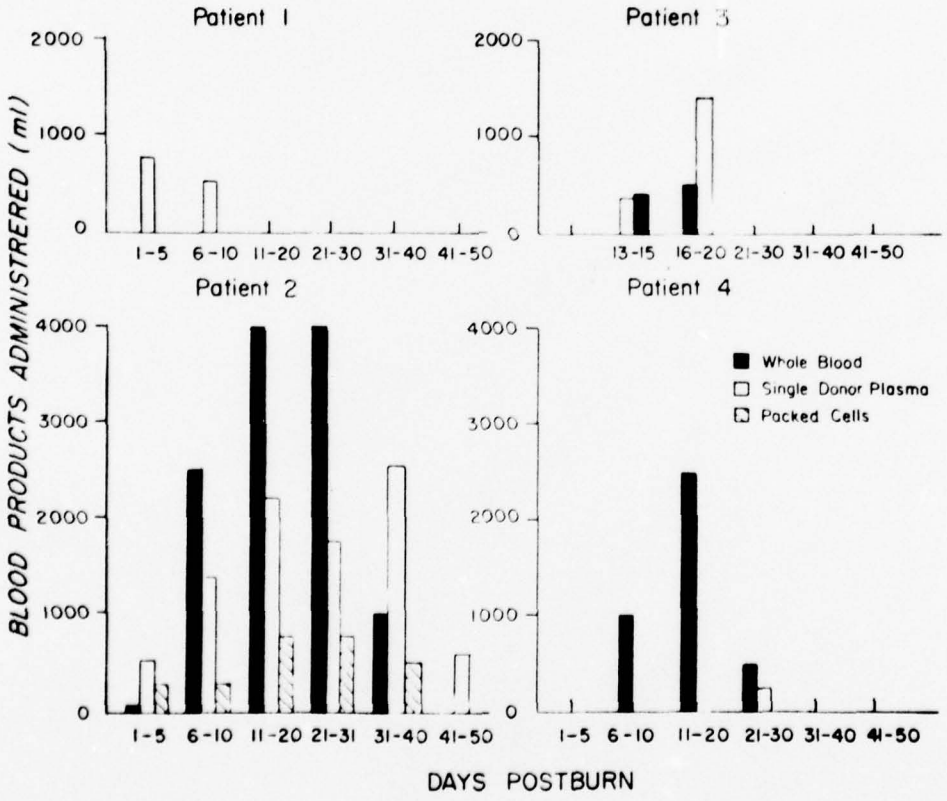


Figure 23. Temporal sequence of the administration of blood products to the four non-septic burned patients. The Arabic numerals represent the patient numbers. Refer to Table 7 for the clinical characteristics of the patients.

patients, but only during the first 5 days post admission. C3b INA was also decreased in these patients, and in two of them, occurred only when C3 was reduced concurrently. The decrease in C3b INA appeared to result from increased utilization in cleavage of C3b to its inactive forms. The results suggested that alternative pathway consumption occurring during septicemia in the one patient resulted from the generation of C3b via consumption of the classical pathway. C3b together with factors B and D, and properdin would lead to further depletion of C3 and C5 via the enzymes, $\overline{C3b,B}$ and $\overline{C3b,B,P}$. This observation provides an explanation for the results of our earlier study, showing that both complement pathways were consumed in one septic burned patient, and only the classical pathway was consumed in the other septic patient (9). The results do not exclude the possibility that the alternative pathway was consumed directly as the result of interaction of the bacteria with early components in this pathway rather than through the feedback mechanism. Although if this were the case, then one would expect that alternative pathway consumption would have occurred more frequently in the septic patients and would not necessarily have been associated with marked classical pathway consumption.

Reduction in classical pathway activity was demonstrated in all of the septic burned patients during the initial postburn period. Total hemolytic complement (CH_{50}) was always markedly reduced below the normal mean value for the non-septic patients, and early complement components were reduced as well. In four patients, the reduction included C1 to C5, in another, only C1 and C4, and in the other two, C1, C4, and C2 and C5 or only C1, C4, and C2. CH_{50} and the early classical components were reduced not only during the initial postburn period, but during the time prior to when the first positive blood culture was obtained. The reduction in classical pathway activity was not found to be the result of systemic infection, since blood cultures were consistently negative in the patients during this time. Reduction in classical pathway activity was not demonstrated in the non-septic burned patients, suggesting that this humoral abnormality is predictive of septic episodes. This is an extremely exciting concept and, if substantiated, could lead to the early identification of high risk patients and possible alterations in therapeutic approaches.

The lack of demonstration of classical complement pathway consumption during the study period in the non-septic burned patients could not be explained on the basis of the administration of blood products, since these patients as a group received less whole blood and plasma than the septic burned patients. The administration of blood products also did not influence the outcome of septicemia, since the patient with the most prolonged septicemia received the largest amounts of blood products per unit of time. In addition, the two other patients who died of septic shock received amounts of whole blood and plasma per unit of time roughly equal to the non-septic patient who received the most blood products.

More abnormalities of C3 and C5 conversion by CoVF and/or inulin were observed in the septic burned patients than in the non-septic burned patients. However, there was quite a discrepancy in the average burn ratio (% total injury/ % third degree injury) between the two groups (55/43 for the septic patients

and 49/19 for the non-septic patients). The increased number of abnormalities in the septic population is probably explained by this finding, since reduction in C3 conversion has been shown to be a function of increasing burn size (Two of the three burned patients who died of septic shock had reduced C3 and C5 conversion for the entire study period (Patient III) or prior to the development of septic shock (Patient V), during which time C3 and C5 conversion were normalized. In the other patient (#VI), C3 and C5 conversion were normal prior to the development of septic shock and became reduced during shock. In the surviving patients, C3 conversion was often reduced when C5 conversion was normal and vice versa. These observations were also documented in three of four non-septic burned patients. The reason for the lack of an exact correlation between C3 and C5 conversion in the burn sera was not addressed by our investigation, but will be the subject of future study. However, the most important observation to be derived from the C3 and C5 conversion data is that there was absolutely no correlation between these abnormalities and the occurrence, duration, or outcome of septicemia.

As outlined in the previous section of this report (1a), C3 conversion by the activating substances was a function of the alternative complement pathway, and C5 conversion was a function of both the classical and alternative pathways. Values for concentrations and conversion of C3 and C5 were consistently higher when measured by hemolytic assays, in comparison to the results obtained from the immunochemical determinations. There was a marked variability in the results of the hemolytic assays, presumably because determinations were run with different lots of commercial EAC$\overline{14}$ cells and purified human components, C2, C6, C7, C8, C9, and C3 or C5. In all future experiments, determinations will be run utilizing the same lots of erythrocytes and complement components.

The classical pathway appeared to be activated preferentially in all but one of the burned patients during septic episodes. Although C3 and/or C5 conversion were often reduced during septic episodes, levels of factor B were generally normal or elevated. Since it is well known that factor B is consumed during alternative pathway activation, the results suggested that the alternative pathway was reduced in the burned patients due to blockage of this pathway rather than to consumption of alternative pathway components. Blockage of the alternative pathway resulting from deficient alternative pathway proteins would provide an explanation for the observed preferential activation of the classical pathway occurring during septicemia. Further studies regarding evidence for a deficiency of alternative pathway components causing decreased C3 conversion in burned patients is presented in section A1 of this report.

Consumption of components of the alternative and/or classical complement pathway did not decrease the opsonic capacity of the patients' sera for their own infecting microorganisms. Multiple bacteria were isolated from the septic burned patients including S. aureus, E. coli, P. aeruginosa, K. pneumoniae, C. freundii, and S. faecalis. It should be emphasized that none of the infecting microorganisms was susceptible to direct lysis by normal or burn sera or to phagocytosis and intracellular killing by normal PMNs in the absence of the sera. The concentration of serum used in the opsonic assays was specific for each infecting microorganism. Concentrations of the patients' sera were based

on the minimal concentration of pooled normal human serum which was found to promote maximal intracellular killing of each bacterial strain during the shortest incubation period.

The finding that complement consumption did not reduce the opsonic capacity of the patients' sera for their infecting microorganisms is most intriguing. The investigators offer three possible explanations for this observation as follows: (a) Only minute amounts of classical or alternative complement pathway components are required for effective phagocytosis and intracellularly killing of the infecting microorganisms; (b) immune antibodies may be produced during the infection which either alone or in combination with minimal levels of classical complement components effectively opsonize the infecting microorganisms; or (c) naturally occurring antibodies to the infecting microorganisms may be present in the burn sera prior to the infection which together with minimal levels of alternative and/or classical complement pathway components effectively opsonize the microorganisms. Data are presented in section C1 of this report which show that under normal conditions the majority of gram-negative aerobic bacilli isolated from burned patients require immunoglobulin and utilize the alternative and/or classical complement pathway during the opsonic process. Therefore, either antibodies must be present in the burn sera prior to infection or be produced during colonization with the microorganisms, if effective opsonization in the presence of minimal levels of complement components is to occur. Our future studies will be directed toward determining the levels of pre-existing antibodies and antibodies produced during the course of the infection to the infecting microorganisms in the septic burned patients. The septic burned patient represents a new model for studying the in vivo role of complement and immunoglobulins in opsonization of opportunist microorganisms.

B. Occurrence and Duration of Changes in Humoral Components of Host Defense in Patients with Abdominal Trauma and in Surgical Patients without Trauma

1. Results

Our previous studies showed that abnormalities of both the classical and alternative complement pathways occurred immediately following severe blunt or penetrating abdominal trauma (25). Conversion of C3 by cobra venom factor (CoVF), which is a functional measurement of the alternative complement pathway, was reduced in the sera of ten trauma patients, and decrease in the level of properdin and C3b inactivator (C3b INA) was also demonstrated in the trauma sera. Total hemolytic complement (CH_{50}), a functional measurement of the classical complement pathway and the immunochemical level of C5 were also decreased. Conversion of C3 by inulin and levels of factor B, C1q, C4, C2, and C3 were not found to be statistically decreased in the patients' sera. The concentration of IgM was significantly reduced in the sera of the trauma patients, although levels of IgG and IgA were not abnormal. The present investigation was undertaken to determine the cause and significance of the humoral abnormalities in the trauma patients and to determine the duration of the abnormalities.

Our first objective was to determine if reduction in serum opsonizing activity was associated with decrease in complement activity or IgM concentration in the previously studied group of ten trauma patients. The published original data of the complement levels and activities and immunoglobulin levels in the sera of the ten trauma patients are shown in Table 8. Opsonic activity for E. coli 075, P. aeruginosa 73044, and S. aureus 502A was measured in the patients' sera. Opsonic activity for the E. coli and S. aureus strains was significantly reduced ($p = < 0.05$), in comparison to the opsonic activity of sera from age-matched normal individuals (Figure 24). Opsonic activity for the P. aeruginosa strain was markedly reduced in the sera of two of the patients, however the overall differences in opsonic activity for P. aeruginosa between the patients and the controls were not significant ($p = < 0.1$). The serum from one of the patients with reduced opsonic activity for P. aeruginosa also had reduced opsonic activity for the E. coli and S. aureus test strains. However, a significant correlation by single regression analyses of opsonization of the sera for the three strains was not demonstrated. Moreover, the only correlation between opsonic activity for the test strains and immunoglobulin and complement levels or complement activities was observed with the E. coli strain. Opsonic activity of the sera for the E. coli strain correlated with C3 conversion by inulin ($r = 0.92$; $p = 0.0001$) and with C3 concentration ($r = 0.65$; $p = 0.042$). The results indicated that reduction in serum opsonic activity for E. coli 075 was a function of the alternative complement pathway, but that reduction in opsonic activity for the S. aureus and P. aeruginosa strains was related to something other than classical or alternative pathway activity or total immunoglobulin level. The observation that the sera from the trauma patients had reduced opsonizing activity for three microorganisms which frequently infect trauma patients provides evidence that humoral host defense mechanisms in patients with abdominal trauma are compromised.

Our next experiments were directed toward determining the occurrence and duration of the humoral abnormalities in patients with penetrating abdominal trauma and the association between the abnormalities and microbial infections. Clinical characteristics of the study patients and information regarding cultures obtained on the patients are shown in Table 9. Levels and activities of components of the classical and alternative complement pathways, levels of IgG, IgA, and IgM, and serum opsonic activity for E. coli 075, P. aeruginosa 73044, and S. aureus 502A were measured as soon as the patients were identified, and then twice weekly until the patients were discharged.

In all of the patients, total hemolytic complement (CH_{50}) was reduced below the normal mean value during the first 48 hours post trauma and was restored to or above the normal mean value in only two of the patients (#4 and 6) during the 26 day post trauma study period; restoration of CH_{50} to normal in Patients 4 and 6 occurred by the end of the first week post trauma (Figure 25). During the first 48 hours post trauma, C1q, C4, C2, C3, and C5 were reduced concurrently below normal mean values in Patients 1 and 2. Patient 3 had reduced C3 during the first 48 hours post trauma. C3, C4, and C5 were reduced in Patients 4 and 5; C1q was also reduced in Patient 5, but not in Patient 4. In Patient 6, only C5 was reduced, and in Patient 7, C1q, C3, and C5 were decreased during the first 48 hours post trauma. C1q and C5 remained reduced in Patient 5 for the duration of the study. Restoration of the classical components to a normal mean value

Table 8. Immunochemical and Functional Activities of Components of the Alternative and Classical Complement Pathways, Immunoglobulin Levels, and Opsonization in the Sera of Ten Patients with Abdominal Trauma[a]

Patient No.	CH_{50} μ/ml	C1q % of N[b]	C4 % of N[b]	C2 % of N[b]	C3 % of N[b]	C5 % of N[b]	Be % of N[b]	C3b INA % of N[b]	P[f] % of N[b]	C3 Conversion % I[c]	C3 Conversion % CoVF[d]	IgM mg%	IgG mg%	IgA mg%
1	26	52	75	64	100	47	87	50	65	67	87	98	2300	220
2	27.5	80	87	69	90	65	81	56	60	69	83	52	2000	72
3	27.5	48	64	50	73	56	73	32	70	71	87	24	1750	64
4	25.5	100	76	85	120	90	157	68	67	80	76	58	3350	386
5	28	80	116	55	100	96	110	45	82	61	79	62	2800	194
6	8.4	42	56	55	70	41	110	45	50	79	87	106	2200	140
7	29	116	100	85	113	106	143	63	72	70	76	112	3350	340
8	18	42	45	55	56	43	81	25	60	0	53	118	2100	96
9	26	66	83	55	70	65	66	50	73	24	91	37	2600	176
10	25	95	91	96	120	76	130	71	66	54	80	70	3500	128

[a]Sera were obtained from the patients within 72 hours following the injury.

[b]N = normal.

[c]I = inulin.

[d]CoVF = cobra venom factor.

[e]B = factor B.

[f]P = properdin.

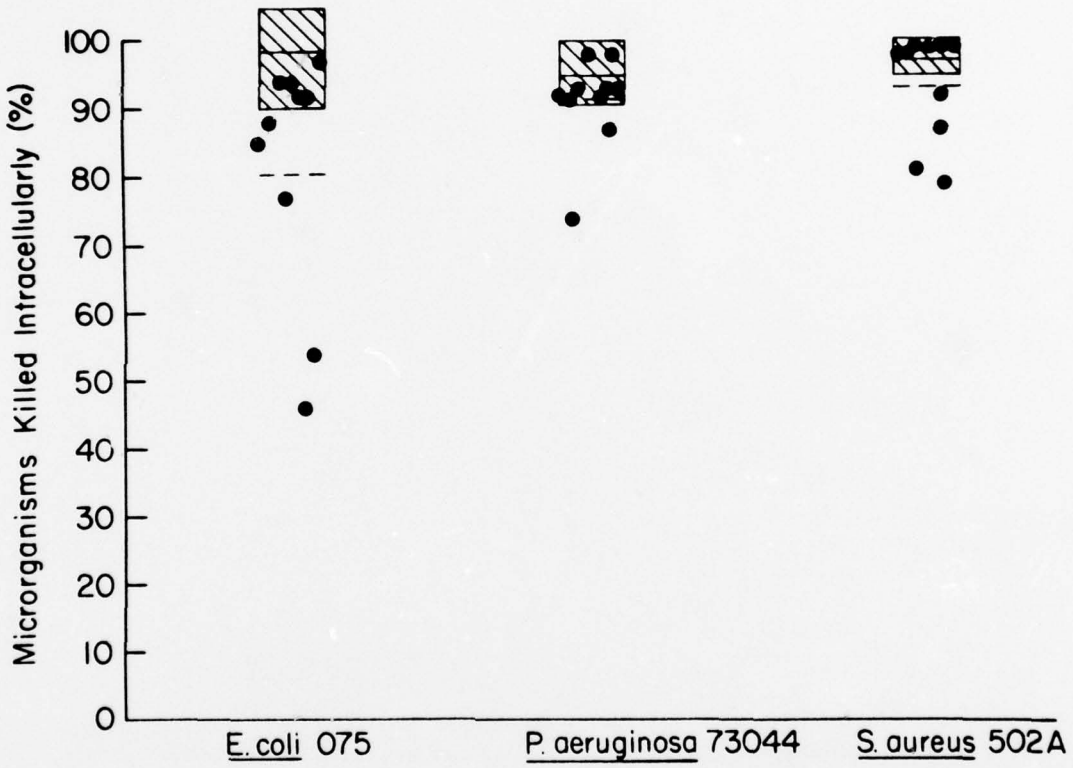


Figure 24. Opsonic activity for E. coli 075, P. aeruginosa 73044, and S. aureus 502A in the sera of ten patients with abdominal trauma. The dots represent values for each patient, and the dotted horizontal lines represent the mean values for the patient group. The shaded areas represent the normal ranges (mean $\pm$ 2 S.D.) for each determination. The solid horizontal lines represent the normal mean values. Statistical analysis was performed by the Student t test.

Table 9. Clinical Characteristics of the Patients with Abdominal Trauma

Patient No.	Age	Sex[a]	Site of GSW[b]	Positive Cultures		
				Specimen	Microorganisms	Day of Positive Culture[d]
1[c]	27	M	abdomen	abdominal wound drainage	P. aeruginosa B. fragilis S. epidermidis	8
				blood	diphtheroids B. fragilis	16
				pleural cavity pus	B. fragilis	16,19,22
2	15	M	abdomen	blood	diphtheroids	7
				abdominal wound drainage	diphtheroids S. epidermidis	14
3	18	M	abdomen	none		
4	50	M	chest,neck, abdomen	none		
5	21	F	abdomen	none		
6[c]	19	M	abdomen	none		
7	17	M	abdomen	none		

[a]M = male; F = female.

[b]GSW = gun shot wound.

[c]Splenectomized on the day of trauma.

[d]Refers to day post trauma that the positive culture was obtained.

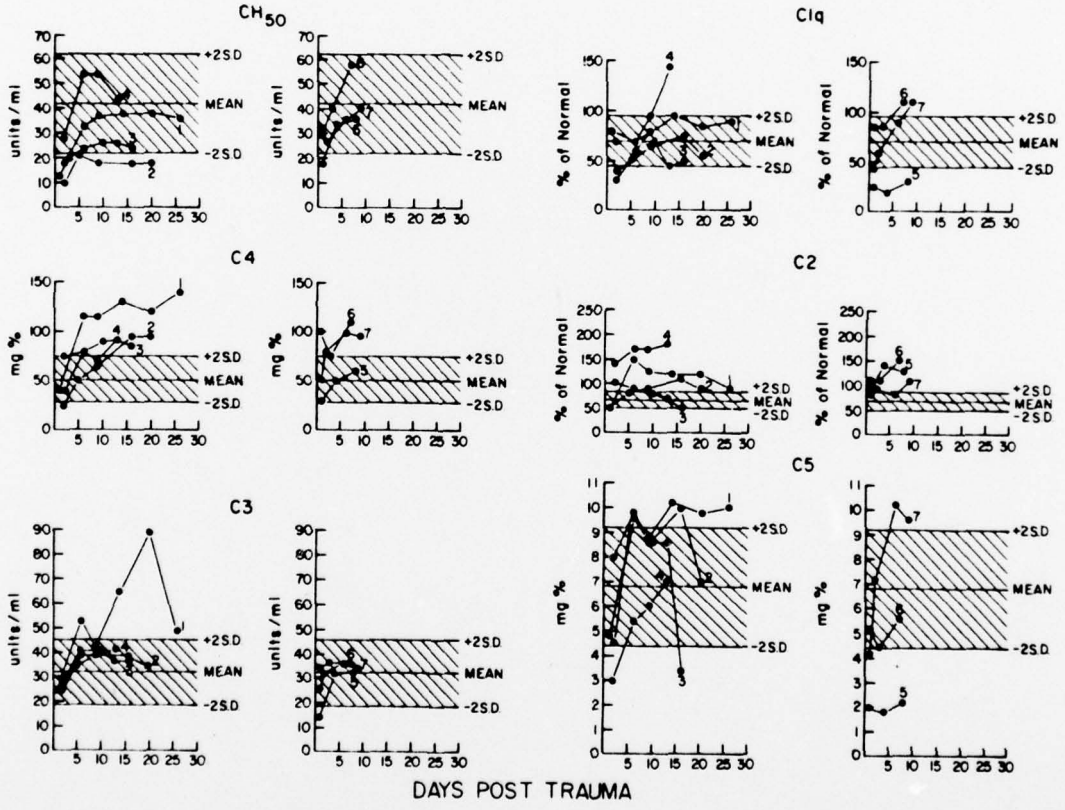


Figure 25. Immunochemical levels and functional activities of components of the classical complement pathway in the sera of seven patients with abdominal trauma during 26 days post trauma. The shaded areas represent the variation in 20 individual normal sera (mean $\pm$ 2 S.D.). Numbers following the lines represent patient numbers. Refer to Table 9 for clinical characteristics of the patients. Mean values of duplicate or triplicate determinations are presented.

occurred in all of the other patients. The components remained normal for the duration of study in all of these patients, except Patient 3, whose Clq, C2, and C5 became reduced during the second week post trauma.

C3b INA was reduced during the first 48 hours post trauma in Patients 1, 4, 5, 6, and 7 and was restored to normal thereafter (Figure 26). Properdin levels fell below the normal mean value for the duration of the study only in these five patients. In addition, C3 conversion by CoVF and/or inulin fell below normal mean values during the study in these patients. Factor B levels were generally normal or elevated in the sera of all of the patients for the entire study period.

IgG levels were markedly decreased during the study in Patients 4 and 5; IgG was also reduced initially in Patient 1 (Figure 27). IgA and IgM levels in all of the patients fell within normal ranges; however, IgM was decreased initially in Patients 2, 3, 4, 5, and 6 and IgA fell below the normal mean value in Patient 6 during the study.

Serum opsonization for S. aureus 502A was reduced in Patient 4 for the duration of the study (Figure 28). Serum opsonization for E. coli 075 was only reduced during 48 hours post trauma in Patient 5. Serum opsonization for P. aeruginosa 73044 was reduced in Patient 4 for the duration of the study, and intermittently in Patients 1, 2, 6, and 7. The observation that Patient 4 had reduced opsonizing activity for P. aeruginosa and S. aureus could have been related to a deficiency of IgG, but Patient 5, whose serum was deficient in IgG, had normal opsonizing activity for the test strains.

None of the initial decreases in classical or alternative pathway components or activities, immunoglobulin levels, or opsonic activity were related to the levels of total protein in the patients' sera. Further analyses have shown that prolonged decreases in CH_{50}, Clq, C5, IgG and opsonins were also not related to total protein concentrations. These results indicated that the humoral abnormalities were not caused by fluid imbalances.

Two of the seven patients on our study developed post-operative microbial infections (refer to Table 9) associated with initially decreased classical pathway components and activity, but with no other marked humoral abnormalities. One of these patients was the only patient on our study to have persistently decreased classical pathway activity.

We have also measured classical and alternative pathway components and activities, immunoglobulin levels, and opsonic activity for S. aureus 502A, E. coli 075, and P. aeruginosa 73044 in the sera of three surgical patients without trauma pre-operatively and during a 3 week post-operative period. In these patients, we have demonstrated no abnormalities of complement, immunoglobulins, or opsonins.

2. Discussion

Because of the small number of patients on our study, generalizations about humoral abnormalities in patients with abdominal trauma cannot be made at this

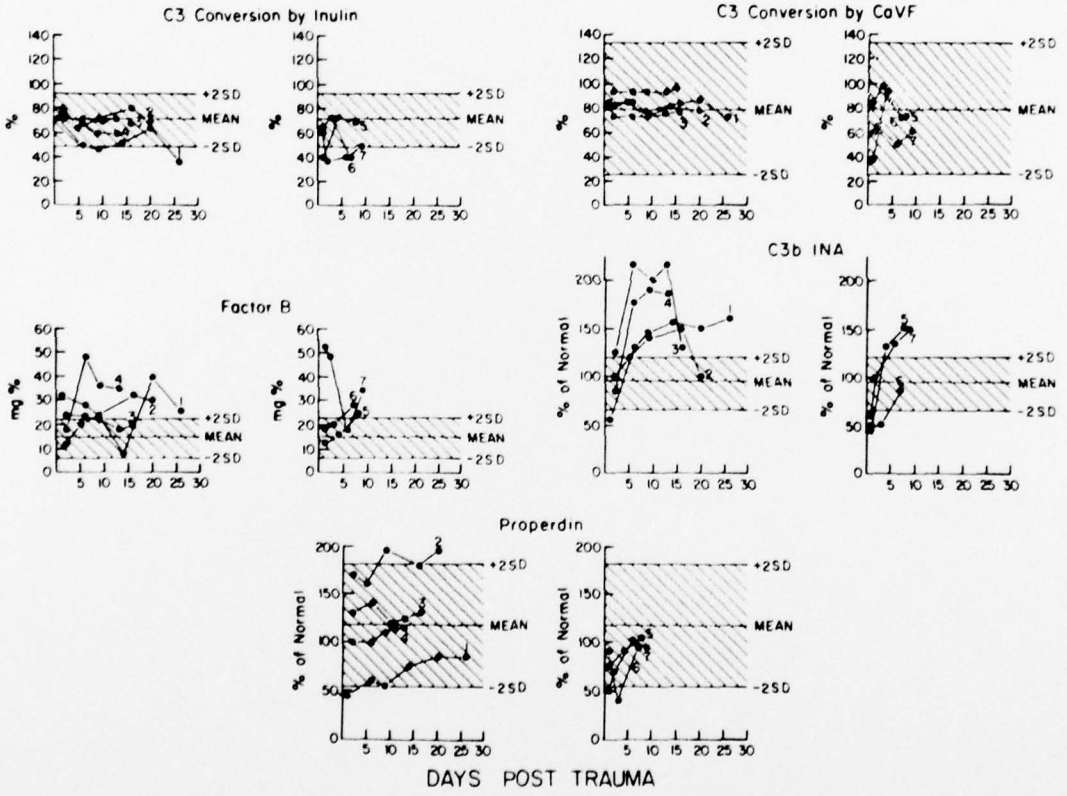


Figure 26. Immunochemical levels and functional activities of components of the alternative complement pathway in the sera of seven patients with abdominal trauma during 26 days post trauma. The shaded areas represent the variation in 20 individual normal sera (mean $\pm$ 2 S.D.). Numbers following the lines represent patient numbers. Refer to Table 9 for clinical characteristics of the patients. Mean values of duplicate or triplicate determinations are presented.

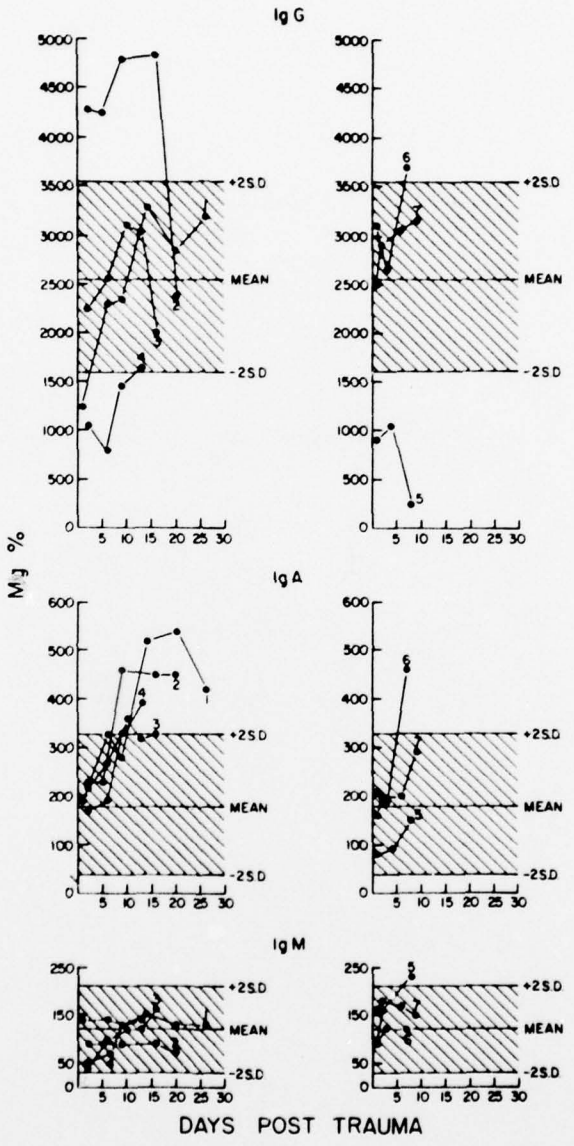


Figure 27. Immunochemical levels of IgG, IgA, and IgM in the sera of seven patients with abdominal trauma during 26 days post trauma. The shaded areas represent the variation in 20 individual normal sera (mean $\pm$ 2 S.D.). Numbers following the lines represent patient numbers. Refer to Table 9 for clinical characteristics of the patients. Mean values of duplicate or triplicate determinations are presented.

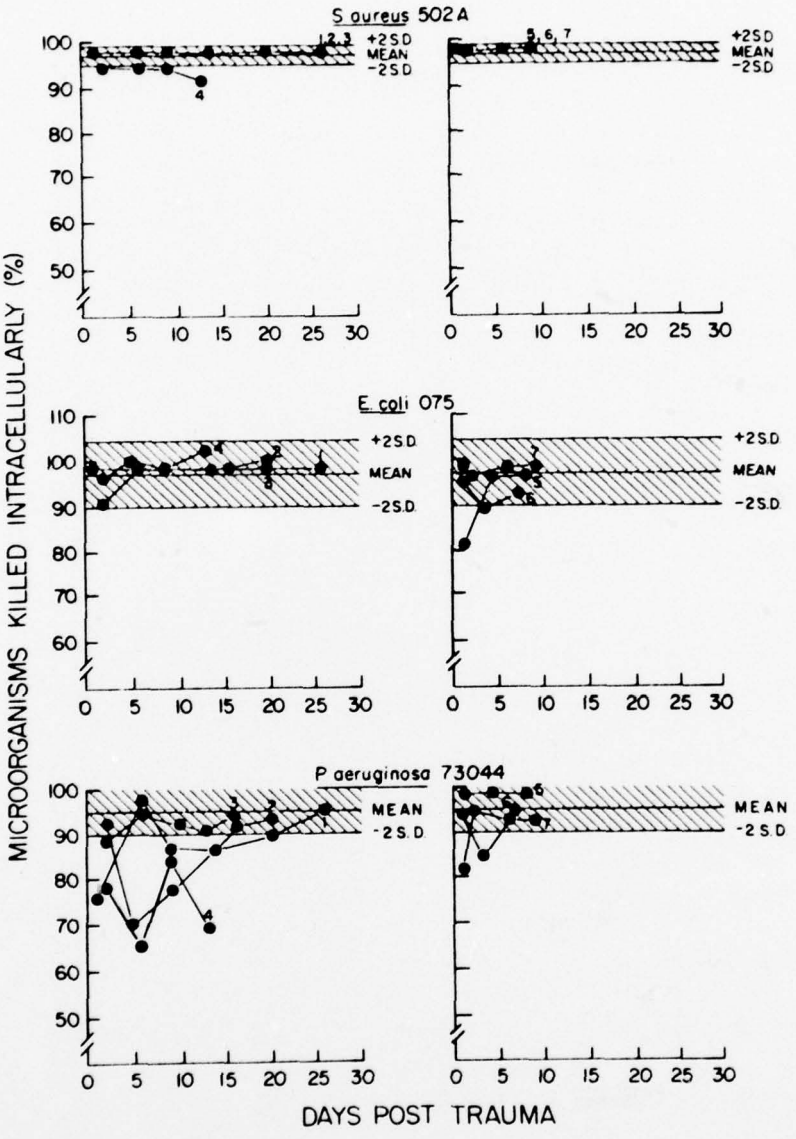


Figure 28. Opsonic activity for E. coli 075, P. aeruginosa 73044 and S. aureus 502A in the sera of seven patients with abdominal trauma during 26 days post trauma. The shaded areas represent the variation in 20 individual normal sera (mean ± 2 S.D.). Numbers following the lines represent patient numbers. Refer to Table 9 for clinical characteristics of the patients. Mean values of duplicate or triplicate determinations are presented.

time. Our investigation has, however, permitted several preliminary conclusions to be drawn as follows: (a) Multiple abnormalities of the classical and alternative complement pathways, immunoglobulins, and opsonins were shown to occur following abdominal trauma, some of which persisted after the first week post trauma; (b) the humoral abnormalities in the trauma patients were not related to fluid imbalances or to surgical procedures; (c) reduction in IgM occurring during the initial post trauma period was not related to splenectomy, since patients who had not undergone splenectomy had decreased IgM and one of the splenectomized patients had normal IgM; (d) decrease in IgG, in addition to IgM, was demonstrated in the second patient study group; (e) decreases in C1q, C4, C2, C3, and C3 conversion by inulin were demonstrated in the second study group but not in the first; (f) patients who subsequently developed microbial infections were the only patients who had decreased classical pathway activity initially that appeared to result from consumption of components, since only in these patients were C1q, C4, C2, C3, and C5 decreased concurrently; (g) cause-and-effect relationships were not established between the abnormalities, with the exceptions that E. coli 075 opsonization correlated with alternative pathway activity, and decrease in C5 appeared to correlate with decrease in C3b INA; and (h) decreases in classical or alternative pathway components or immunoglobulins G, A, or M did not appear to correlate with opsonization for S. aureus 502A or P. aeruginosa 73044 in either study group.

The observation that E. coli 075 opsonization in the nonburn trauma patients correlated with alternative pathway activity supports the concept that the alternative pathway is utilized preferentially in opsonization of this bacterium (2). The finding that correlation between E. coli 075 opsonization and classical pathway activity occurred in patients with burn trauma provides further support to the hypothesis that the classical pathway is utilized in opsonization secondarily and only if the alternative pathway is blocked.

The number of patients on our study did not equal the number of patients we had projected to study (ten abdominal trauma, ten head trauma, ten surgical nontrauma). This was caused by multiple problems we encountered throughout the year. Our greatest problem was in locating surgical patients at the Cincinnati General Hospital who did not have underlying diseases which would disqualify them for our study. Our second most difficult problem was in obtaining informed consent from the relatives of our abdominal trauma patients. Most of the trauma in this population of patients was either caused by family members who had been incarcerated and therefore could not or would not give permission, or the family members could not be located. An additional problem was in obtaining a population of individuals who had sustained head trauma without abdominal trauma. We were unable to identify any patients with head trauma without blunt abdominal trauma who survived. Our final problem related to the percent of effort and support we had designated for our research nurse and clinical investigators. In order to make all of the necessary arrangements with the various attending physicians, identify the patients and obtain permission, follow the patients utilizing the detailed flow sheets and obtain the appropriate samples, Dr. S. Bjornson spent 10 to 15 hours per week on the study rather than 5, and Ms. Geri Perkins, research nurse, spent 75% effort rather than 50%.

C. Normal Human Serum Opsonins for Opportunist Microorganisms

1. Studies to determine the requirements for immunoglobulin and the alternative and classical complement pathways for opsonization of gram-negative aerobic microorganisms

a. Results

Evidence regarding the requirements for immunoglobulin and complement for opsonization of gram-negative and gram-positive aerobic microorganisms has been controversial. Williams and Quie provided evidence that immunoglobulin was required for phagocytosis of Escherichia coli, Pseudomonas aeruginosa, and Proteus vulgaris, but not for phagocytosis of Staphylococcus epidermidis, Serratia marcescens, Streptococcus viridans, and Streptococcus faecalis (57). Jasin showed that immunoglobulin and the classical complement pathway were required for optimal phagocytosis of S. aureus, and that phagocytosis of E. coli occurred via the alternative complement pathway in the absence of immunoglobulin (17). Immunoglobulin in addition to alternative pathway proteins was shown to participate in phagocytosis of P. aeruginosa and Streptococcus pneumoniae (54-56). Subsequent studies showed that S. pneumoniae, S. faecalis, S. viridans, S. aureus, S. epidermidis, S. marcescens, and E. coli required the alternative complement pathway for phagocytosis, but that P. aeruginosa did not (70,71).

Minimal information is available regarding the reasons for the diversity in the requirements for immunoglobulin and the alternative and classical complement pathways for phagocytosis of strains of the same genera. One possibility is that differences in the methodology for measuring phagocytosis produced differences in the results. An alternative explanation is that differences in the requirements for immunoglobulin and complement for phagocytosis exist among strains of the same genera.

The purpose of the present investigation was to determine the requirements for immunoglobulin and complement in human sera for phagocytosis of multiple strains of gram-negative aerobic microorganisms by human polymorphonuclear leukocytes (PMNs). E. coli, Proteus mirabilis, Klebsiella pneumoniae, and S. marcescens were utilized in our study. Most of the microorganisms were isolated from burned patients, although other clinical isolates were included to determine if a difference in the opsonic requirements between the isolates could be demonstrated.

The ability of human sera depleted of immunoglobulin and/or classical complement pathway activity to promote phagocytosis and intracellular killing of the test strains by human PMNs was determined (69). Reaction mixtures consisted of 5.0×10^6 PMNs, 1.0×10^6 bacteria, and serum in a final volume of 1 ml of Hank's balanced salt solution (HBSS). HBSS was substituted for the PMNs or serum in the controls. Serum concentrations used in the assays were based on the minimal amount of pooled normal human serum (PNHS) which promoted maximal intracellular killing of the bacteria during the shortest incubation period.

Hypogammaglobulinemic sera from three different donors (HS_{1-3}) were used as sources of immunoglobulin depleted sera. These sera contained 185 mg % to 475 mg % of IgG, and undetectable levels of IgA and IgM. Hypogammaglobulinemic serum was further depleted of IgG by affinity chromatography using the IgG fraction of rabbit-antihuman IgG coupled to Sepharose 4B; this serum will be referred to hereafter as HS-A. HS-A contained 22 mg % of IgG, and undetectable levels of IgA and IgM. Levels and activities of the classical and alternative complement pathways in HS-A were found to be equivalent to those in PNHS (Table 10).

Table 10. Levels and Activities of the Alternative and Classical Complement Pathways in Hypogammaglobulinemic Serum Further Depleted of IgG by Immunoadsorption

Sera	Total Hemolytic Complement CH_{50} units/ml	C3 (B Antigen) units/ml	C3 Conversion (%)		C3 to C9 CH_{50} units/ml
			Inulin[c]	CoVF[c]	
PNHS[a]	19	30	67	93	31
HS-A[b]	20	30	70	92	24

[a]PNHS = pooled normal human serum.

[b]HS-A = hypogammaglobulinemic serum further depleted of IgG by immunoadsorption.

[c]Inulin and cobra venom factor (CoVF) were the activating substances used for determination of C3 conversion.

Two sources of serum depleted of classical complement pathway activity were utilized. The first source of serum depleted of classical pathway activity was C2 deficient human serum (C2dHS), which was kindly provided by Dr. Paul Quie, University of Minnesota School of Medicine, Minneapolis, Minnesota. The second source of serum depleted of classical pathway activity was PNHS treated with 10 mM ethylene glycol tetra-acetic acid (EGTA) and supplemented with 10 mM $MgCl_2$; this serum will be referred to hereafter as MgEGTA-PNHS. C3 conversion and C3 to C9 consumption by inulin and cobra venom factor in MgEGTA-PNHS and untreated PNHS were equivalent. However, MgEGTA-PNHS was unable to support lysis of sensitized sheep erythrocytes (EA) whereas PNHS supported normal lysis of EA. These results indicated that treatment with EGTA and $MgCl_2$ blocked the classical pathway leaving the alternative pathway intact. In some experiments, individual normal sera or hypogammaglobulinemic sera were treated with 10 mM EGTA and 10 mM $MgCl_2$, and will be referred to hereafter as MgEGTA-N_{1-3} and MgEGTA-HS_{1-3}, respectively. In addition, PNHS depleted of both complement pathways by heating at 56°C for 30 minutes (ΔPNHS) was utilized.

The ability of the various sera to promote phagocytosis and intracellular killing of four different strains of E. coli was first determined. All of the strains were found to be killed by human PMNs in the presence of PNHS, but not by either PMNs or PNHS alone. Hypogammaglobulinemic sera were unable to support phagocytosis of E. coli (A), in comparison to the phagocytosis-promoting activity of PNHS or individual normal sera (N_{1-3})(Figure 29). MgEGTA-PNHS, C2dHS, and ΔPNHS also did not promote phagocytosis of this strain. Similar results were obtained with E. coli (F). Hypogammaglobulinemic sera, MgEGTA-PNHS, C2dHS, and ΔPNHS were unable to promote phagocytosis of this strain, in comparison to normal sera (Figure 30). These results indicated that immunoglobulin and complement were required for phagocytosis and intracellular killing of E. coli (A) and (F), and that the alternative complement pathway was not utilized.

Hypogammaglobulinemic sera and HS-A supported normal phagocytosis of E. coli (P) (Figure 31). MgEGTA-PNHS, MgEGTA-HS_{1-3}, C2dHS, and ΔPNHS were unable to promote phagocytosis of this strain. Similar results were obtained with E. coli (H). Although hypogammaglobulinemic sera and HS-A supported normal phagocytosis of this microorganism, MgEGTA-PNHS, MgEGTA-HS_{1-3}, C2dHS, and ΔPNHS were unable to promote phagocytosis of this strain (Figure 32). These results indicated that the requirement for immunoglobulin was minimal or non-existent for effective phagocytosis of E. coli (P) and (H). Although complement was required for phagocytosis of these strains, the alternative complement pathway was not utilized. In addition, the results indicated that depletion of immunoglobulin and classical pathway activity from human sera was unable to force utilization of the alternative pathway.

Differences in immunoglobulin requirements and complement pathway utilization among three strains of P. mirabilis were also demonstrated. All of the strains were phagocytosed and killed intracellularly in the presence of PNHS and PMNs, but not by either alone. Hypogammaglobulinemic sera, MgEGTA-PNHS, C2dHS, and ΔPNHS did not promote normal phagocytosis of P. mirabilis (H) (Figure 33). These results showed that immunoglobulin and complement were required for phagocytosis of this strain, and that the alternative complement pathway was not utilized.

Hypogammaglobulinemic sera supported phagocytosis of P. mirabilis (A), but showed reduced opsonic activity in comparison to PNHS and individual normal sera

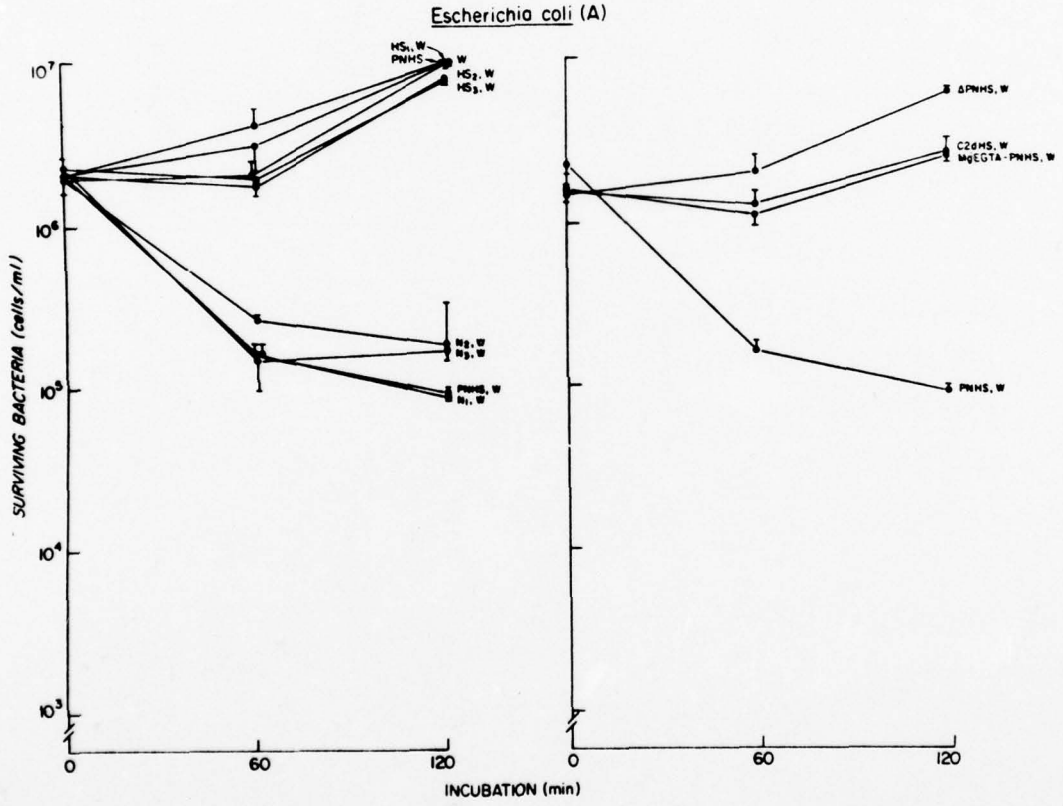


Figure 29. Comparisons of the opsonic activities of normal human sera and sera depleted of immunoglobulin and/or classical pathway activity for E. coli (A). Abbreviations used were as follows: W, polymorphonuclear leukocytes; PNHS, pooled normal human serum; N_{1-3}, individual normal sera; HS_{1-3}, individual hypogammaglobulinemic sera; ΔPNHS, PNHS heated at 56°C for 30 minutes; MgEGTA-PNHS, PNHS treated with 10 mM ethylene glycol tetra-acetic acid (EGTA) and 10 mM $MgCl_2$; C2dHS, C2 deficient human serum. The concentration of sera used in the reaction mixtures was 10%. The points represent mean values of 2 to 4 determinations, and each vertical bar represents the standard error of the mean.

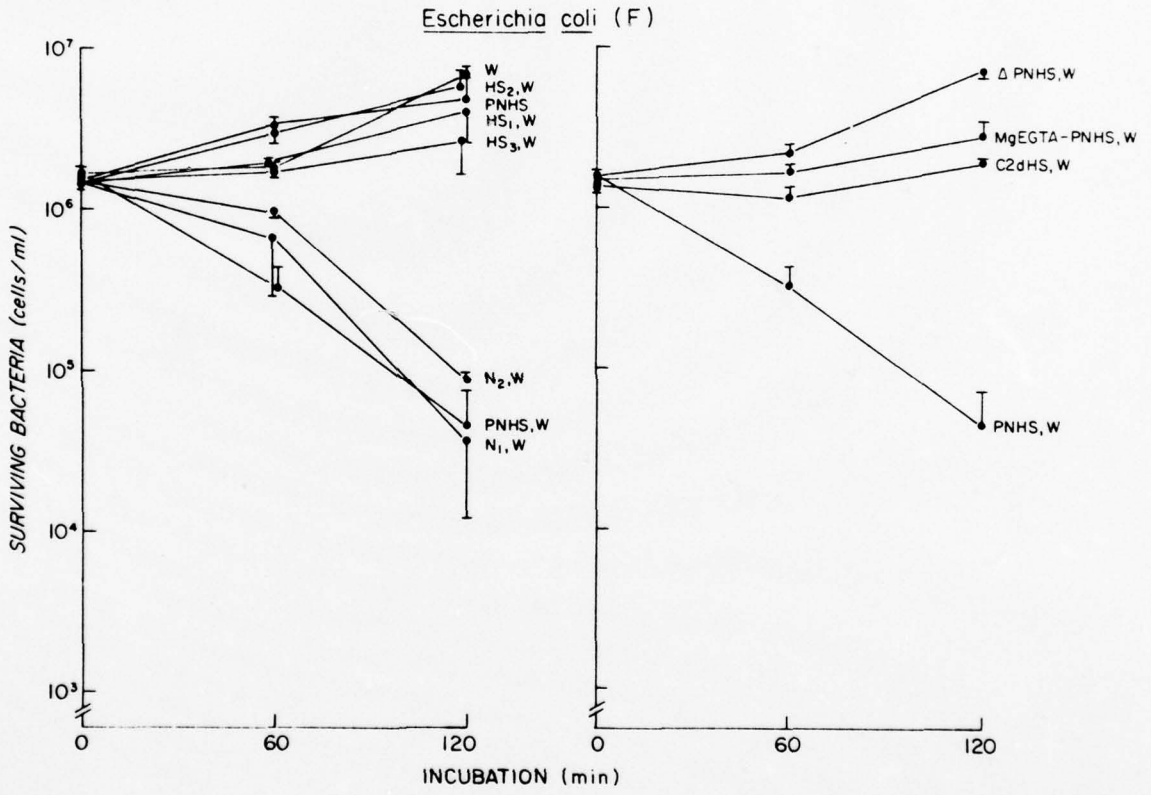


Figure 30. Comparisons of the opsonic activities of normal human sera and sera depleted of immunoglobulin and/or classical pathway activity for E. coli (F). Abbreviations used were as follows: W, polymorphonuclear leukocytes; PNHS, pooled normal human serum; N_{1-2}, individual normal sera; HS_{1-3}, individual hypogammaglobulinemic sera; ΔPNHS, PNHS heated at 56°C for 30 minutes; MgEGTA-PNHS, PNHS treated with 10 mM ethylene glycol tetra-acetic acid (EGTA) and 10 mM $MgCl_2$; C2dHS, C2 deficient human serum. The concentration of sera used in the reaction mixtures was 10%. The points represent mean values of 2 to 4 determinations, and each vertical bar represents the standard error of the mean.

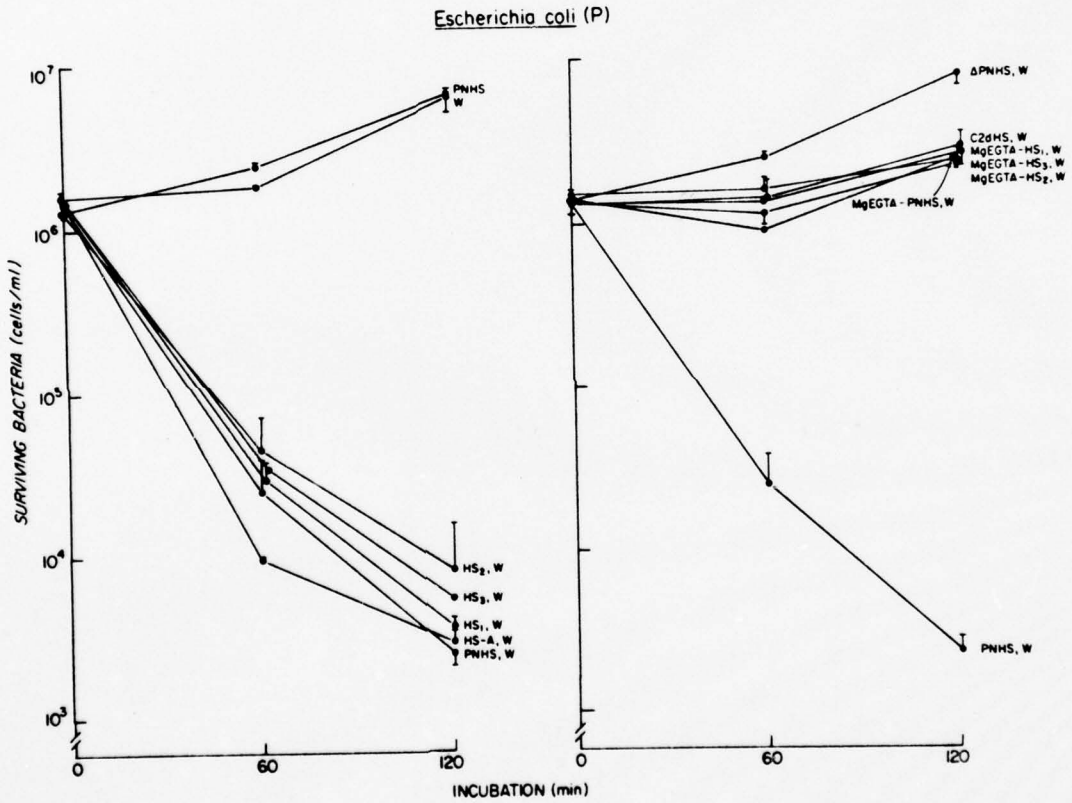
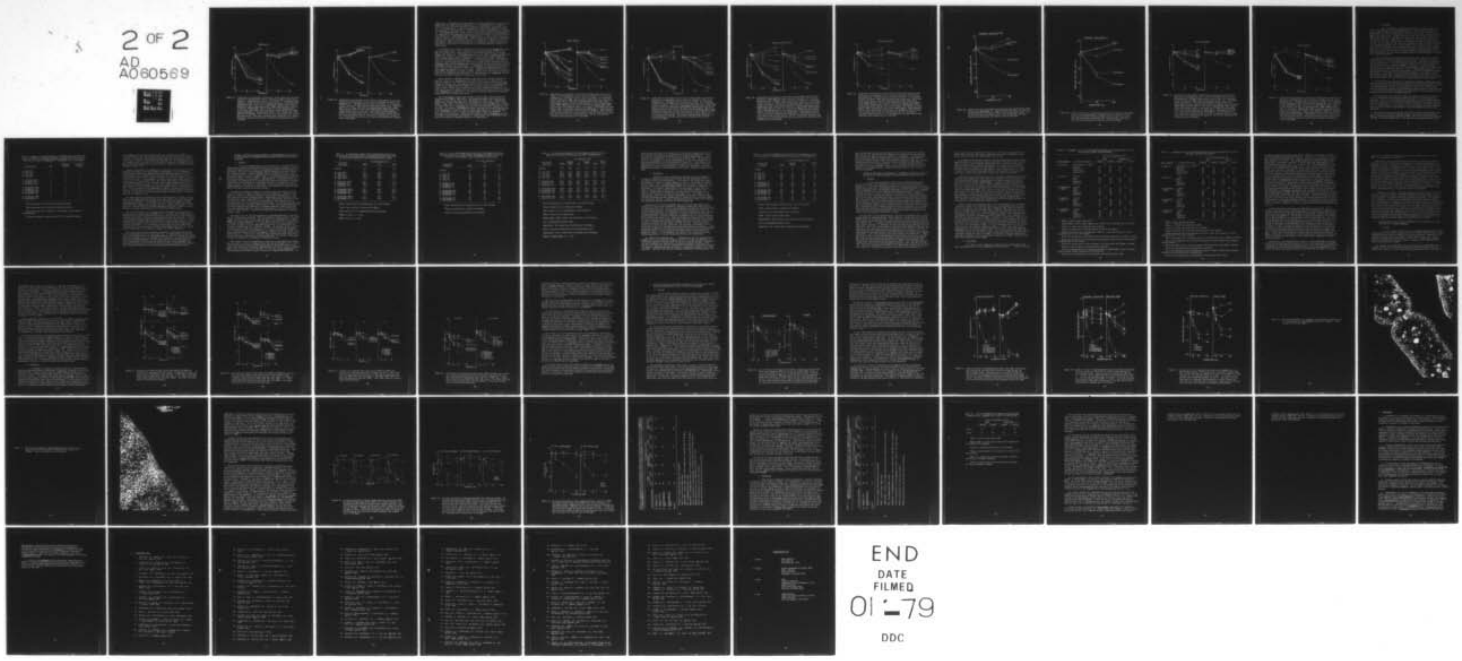


Figure 31. Comparisons of the opsonic activities of normal human serum and sera depleted of immunoglobulin and/or classical pathway activity for E. coli (P). Abbreviations used were as follows: W, polymorphonuclear leukocytes; PNHS, pooled normal human serum; HS_{1-3}, individual hypogammaglobulinemic sera; HS-A, hypogammaglobulinemic serum further depleted of IgG by immunoadsorption; ΔPNHS, PNHS heated at 56°C for 30 minutes; MgEGTA-PNHS, PNHS treated with 10 mM ethylene glycol tetra-acetic acid (EGTA) and 10 mM $MgCl_2$; C2dHS, C2 deficient human serum; MgEGTA-HS_{1-3}, hypogammaglobulinemic sera treated with 10 mM EGTA and 10 mM $MgCl_2$. The concentration of sera used in the reaction mixtures was 5%. The points represent mean values of 2 to 4 determinations, and each vertical bar represents the standard error of the mean.

AD-A060 569 CINCINNATI UNIV OHIO COLL OF MEDICINE F/G 6/13
HOST DEFENSE AGAINST OPPORTUNIST MICROORGANISMS FOLLOWING TRAUM--ETC(U)
JUN 78 A B BJORNSON, W A ALTEMEIER DAMD17-76-C-6023
UNCLASSIFIED NL

2 OF 2
AD
A060569



END
DATE
FILMED
01-79
DDC

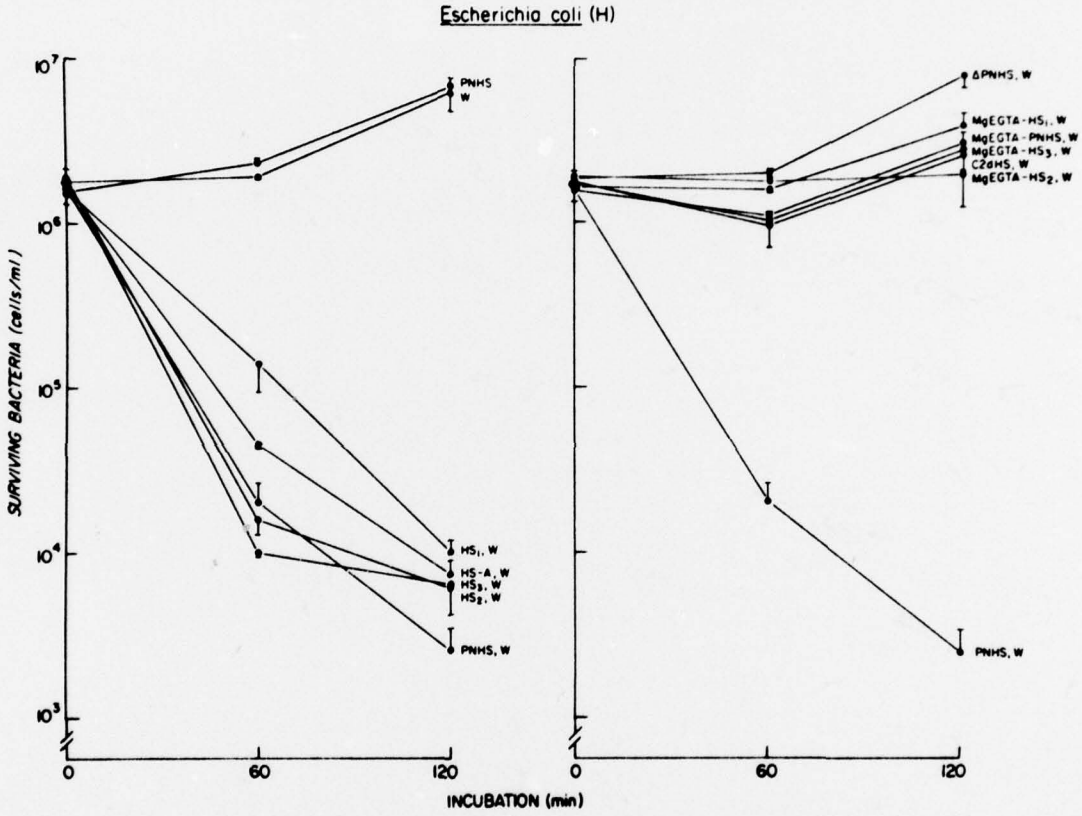


Figure 32. Comparisons of the opsonic activities of normal human serum and sera depleted of immunoglobulin and/or classical pathway activity for E. coli (H). Abbreviations used were as follows: W, polymorphonuclear leukocytes; PNHS, pooled normal human serum; HS_{1-3}, individual hypogammaglobulinemic sera; HS-A, hypogammaglobulinemic serum further depleted of IgG by immunoadsorption; ΔPNHS, PNHS heated at 56°C for 30 minutes; MgEGTA-PNHS, PNHS treated with 10 mM ethylene glycol tetra-acetic acid (EGTA) and 10 mM $MgCl_2$; C2dHS, C2 deficient human serum; MgEGTA-HS_{1-3}, hypogammaglobulinemic sera treated with 10 mM EGTA and 10 mM $MgCl_2$. The concentration of sera used in the reaction mixtures was 5%. The points represent mean values of 2 to 4 determinations, and each vertical bar represents the standard error of the mean.

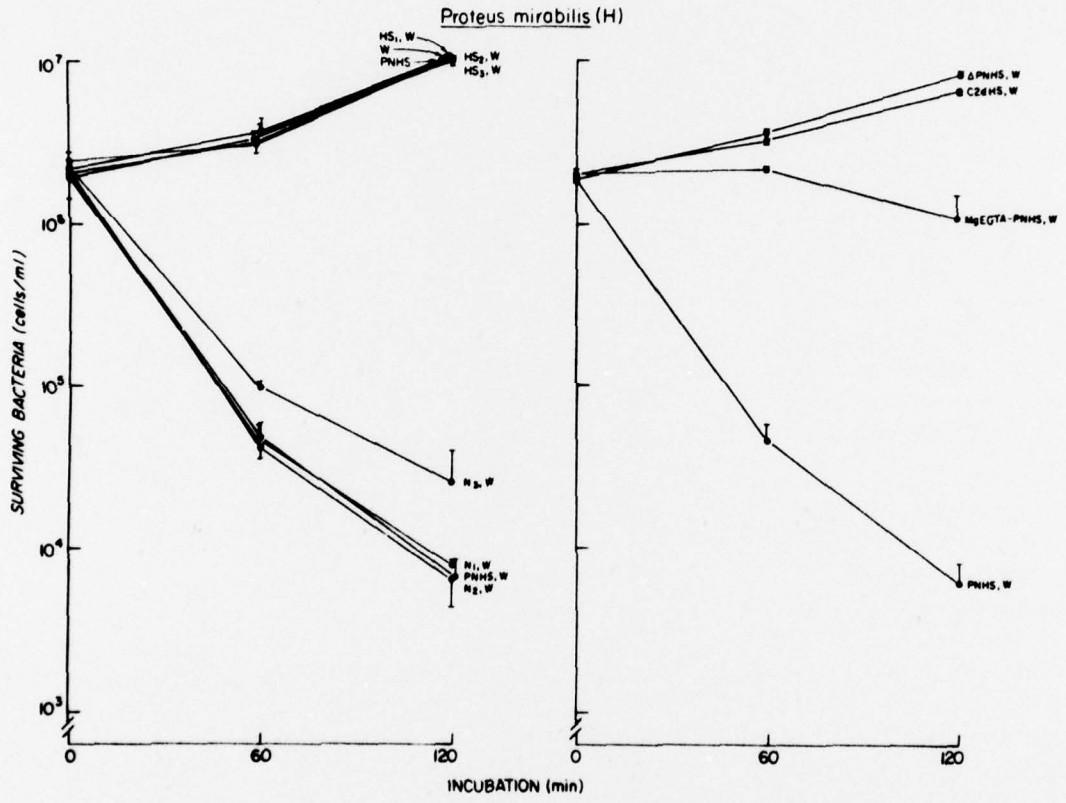


Figure 33. Comparisons of the opsonic activities of normal human sera and sera depleted of immunoglobulin and/or classical pathway activity for P. mirabilis (H). Abbreviations used were as follows: W, polymorphonuclear leukocytes; PNHS, pooled normal human serum; N_{1-3}, individual normal sera; HS_{1-3}, individual hypogammaglobulinemic sera; ΔPNHS, PNHS heated at 56°C for 30 minutes; MgEGTA-PNHS, PNHS treated with 10 mM ethylene glycol tetra-acetic acid (EGTA) and 10 mM $MgCl_2$; C2dHS, C2 deficient human serum. The concentration of sera used in the reaction mixtures was 5%. The points represent mean values of 2 to 4 determinations, and each vertical bar represents the standard error of the mean.

(Figure 34). Furthermore, HS-A was unable to promote phagocytosis of this strain. MgEGTA-PNHS and C2dHS promoted phagocytosis of P. mirabilis (A), although the opsonic activity of these sera was decreased in comparison to the opsonic activity of untreated PNHS. In addition, MgEGTA-HS_{1-3} had reduced opsonic activity, in comparison to untreated HS_{1-3}. Similar results were obtained with P. mirabilis (C). Hypogammaglobulinemic sera did not support phagocytosis of P. mirabilis (C), in comparison to the phagocytosis-promoting activity of PNHS or individual normal sera (Figure 35). MgEGTA-PNHS promoted phagocytosis of this strain, although to a lesser extent than untreated PNHS. C2dHS promoted less phagocytosis than MgEGTA-PNHS, but was similar in opsonic activity to an individual normal serum treated with MgEGTA. These results indicated that P. mirabilis (A) and (C) required immunoglobulin and utilized the alternative and classical complement pathways for phagocytosis.

A diversity in the opsonic capacity of various sera for K. pneumoniae strains was also demonstrated. None of the strains was killed by PMNs or PNHS alone. PNHS and normal sera at concentrations of 5% promoted phagocytosis of K. pneumoniae (Wo) (Figure 36). Hypogammaglobulinemic sera promoted minimal phagocytosis, and HS-A was unable to support phagocytosis of this strain. MgEGTA-PNHS and C2dHS promoted phagocytosis, although not as efficiently as PNHS. MgEGTA-HS_{1-3} were less efficient than untreated HS_{1-3} in promoting phagocytosis, and ΔPNHS was unable to support phagocytosis of this strain. These results indicated that K. pneumoniae (Wo) required immunoglobulin and utilized the alternative and classical complement pathways for phagocytosis. K. pneumoniae (W) was also phagocytosed in the presence of 5% normal sera (Figure 37). Neither hypogammaglobulinemic nor HS-A supported phagocytosis of this strain. Phagocytosis also did not occur with MgEGTA-PNHS, MgEGTA-HS_{1-3}, C2dHS, or ΔPNHS. These results indicated that immunoglobulin and complement were essential for phagocytosis of K. pneumoniae (W), and that the alternative complement pathway was not utilized.

Thirty percent of PNHS was required to promote over a one log reduction in bacterial counts of K. pneumoniae (B) by PMNs (Figure 38). Twenty percent of PNHS was required to achieve a one log reduction in bacterial counts of K. pneumoniae (H) (Figure 39). Although not shown in Figures 38 or 39, hypogammaglobulinemic serum, MgEGTA-PNHS, and ΔPNHS were unable to support phagocytosis of either strain. These results indicated that higher quantitative levels of opsonins were required for phagocytosis and intracellular killing of these two strains of K. pneumoniae, in comparison to the amount of opsonins required for efficient phagocytosis of the other test strains.

Two S. marcescens strains were included in this study; neither strain was phagocytosed by PMNs or PNHS alone. Hypogammaglobulinemic sera and HS-A were unable to promote phagocytosis of S. marcescens (W) (Figure 40). MgEGTA-PNHS, MgEGTA-HS_{1-3}, C2dHS, and ΔPNHS were also unable to promote phagocytosis of this strain. These results indicated that immunoglobulin and complement were required for phagocytosis of S. marcescens (W), and that the alternative pathway was not utilized. In contrast, hypogammaglobulinemic sera and HS-A promoted phagocytosis of S. marcescens (S) equal to that of normal serum (Figure 41). A slight reduction in bacterial counts occurred when MgEGTA-PNHS, C2dHS, and MgEGTA-HS were used as serum sources. These results indicated that S. marcescens (S) had a minimal or non-existent requirement for immunoglobulin and utilized the alternative and classical complement pathways for phagocytosis.

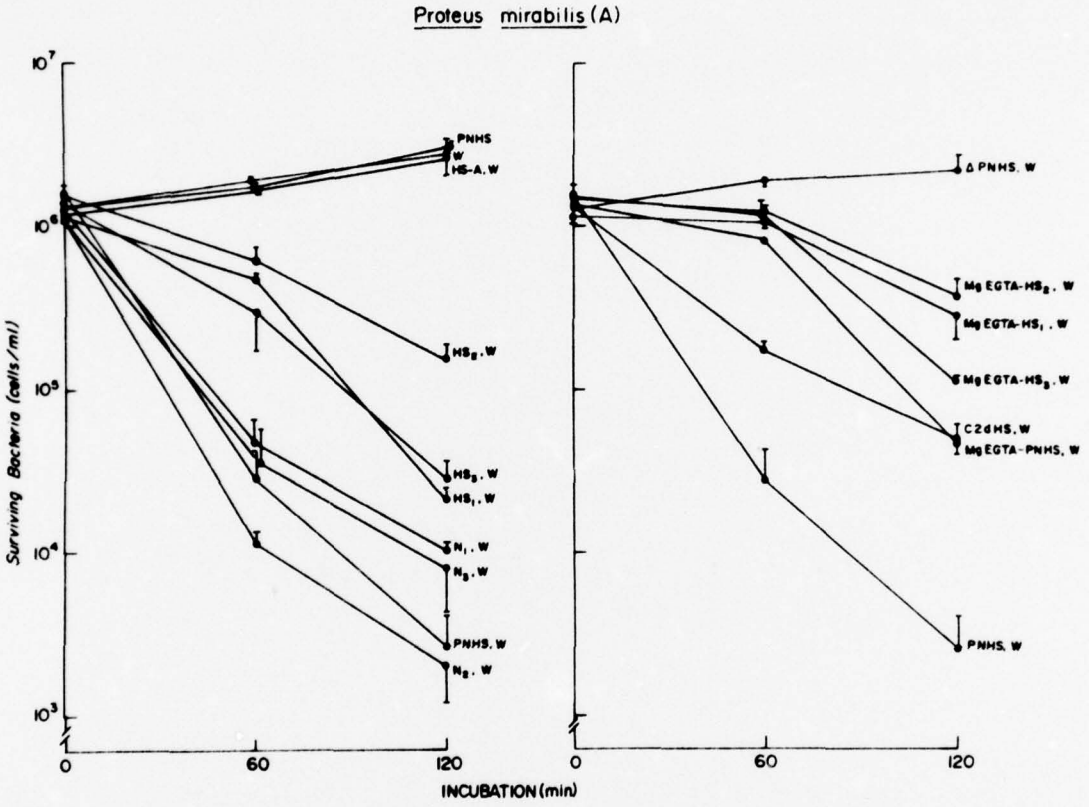


Figure 34. Comparisons of the opsonic activities of normal human sera and sera depleted of immunoglobulin and/or classical pathway activity for P. mirabilis (A). Abbreviations used were as follows: W, polymorphonuclear leukocytes; PNHS, pooled normal human serum, N_{1-3} individual normal sera; HS_{1-3}, individual hypogammaglobulinemic sera; HS-A, hypogammaglobulinemic serum further depleted of IgG by immunoadsorption; ΔPNHS, PNHS heated at 56°C for 30 minutes; MgEGTA-PNHS, PNHS treated with 10 mM ethylene glycol tetra-acetic acid (EGTA) and 10 mM $MgCl_2$; C2dHS, C2 deficient human serum; MgEGTA-HS_{1-3}, hypogammaglobulinemic sera treated with 10 mM EGTA and 10 mM $MgCl_2$. The concentration of sera used in the reaction mixtures was 5%. The points represent mean values of 2 to 4 determinations, and each vertical bar represents the standard error of the mean.

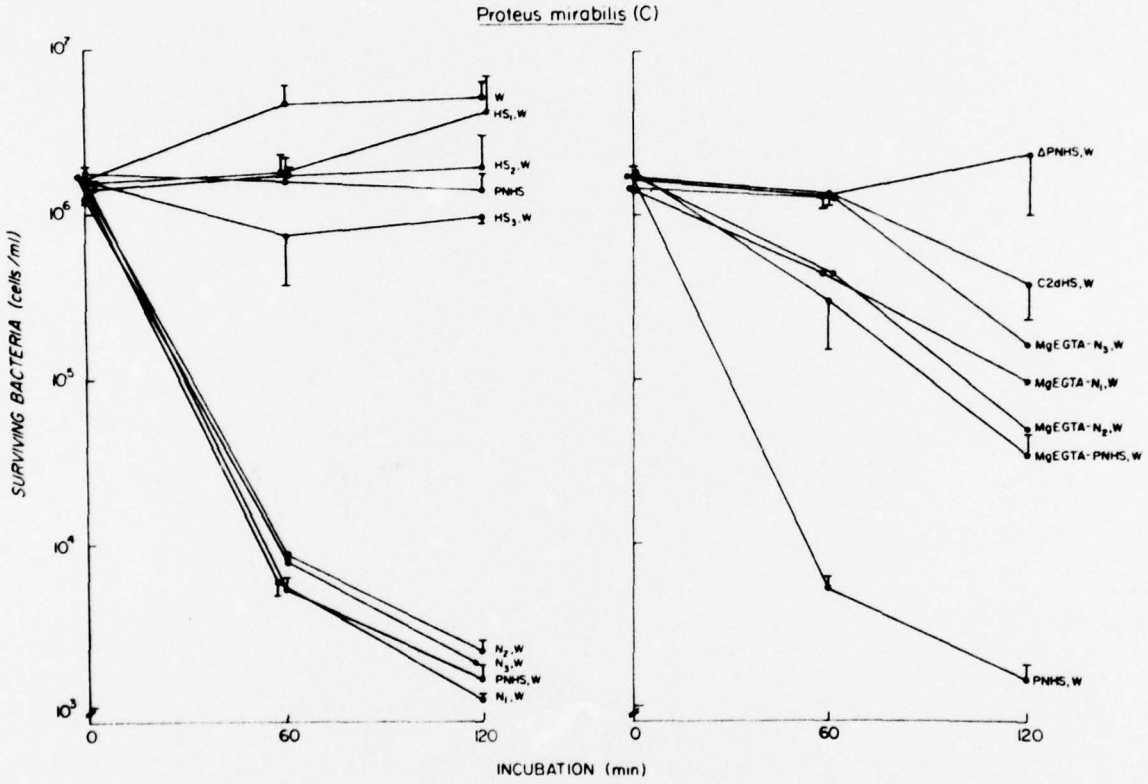


Figure 35. Comparisons of the opsonic activities of normal human sera and sera depleted of immunoglobulin and/or classical pathway activity for P. mirabilis (C). Abbreviations used were as follows: W, polymorphonuclear leukocytes; PNHS, pooled normal human serum; N_{1-3}, individual normal sera; HS_{1-3}, individual hypogammaglobulinemic sera; ΔPNHS, PNHS heated at 56°C for 30 minutes; MgEGTA-PNHS, PNHS treated with 10 mM ethylene glycol tetra-acetic acid (EGTA) and 10 mM $MgCl_2$; MgEGTA-N_{1-3}, individual normal sera treated with 10 mM EGTA and 10 mM $MgCl_2$; C2dHS, C2 deficient human serum. The concentration of sera used in the reaction mixtures was 10%. The points represent mean values of 1 to 4 determinations, and each vertical bar represents the standard error of the mean.

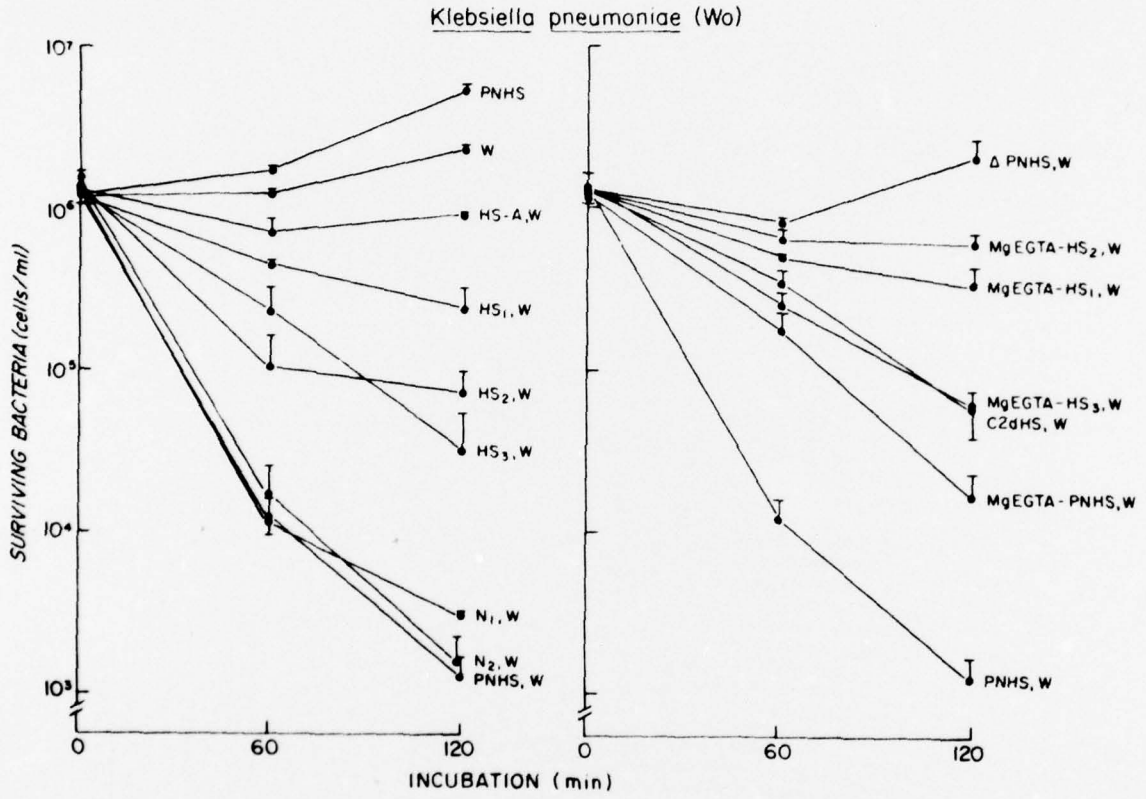


Figure 36. Comparisons of the opsonic activities of normal human sera and sera depleted of immunoglobulin and/or classical pathway activity for K. pneumoniae (Wo). Abbreviations used were as follows: W, polymorphonuclear leukocytes; PNHS, pooled normal human serum; N_{1-2}, individual normal sera; HS_{1-3}, individual hypogammglobulinemic sera; HS-A, hypogammaglobulinemic serum further depleted of IgG by immunoadsorption; ΔPNHS, PNHS heated at 56°C for 30 minutes; MgEGTA-PNHS, PNHS treated with 10 mM ethylene glycol tetra-acetic acid (EGTA) and 10 mM $MgCl_2$; C2dHS, C2 deficient human serum; MgEGTA-HS_{1-3}, hypogammaglobulinemic sera treated with 10 mM EGTA and 10 mM $MgCl_2$. The concentration of sera used in the reaction mixtures was 5%. The points represent mean values of 2 to 4 determinations, and each vertical bar represents the standard error of the mean.

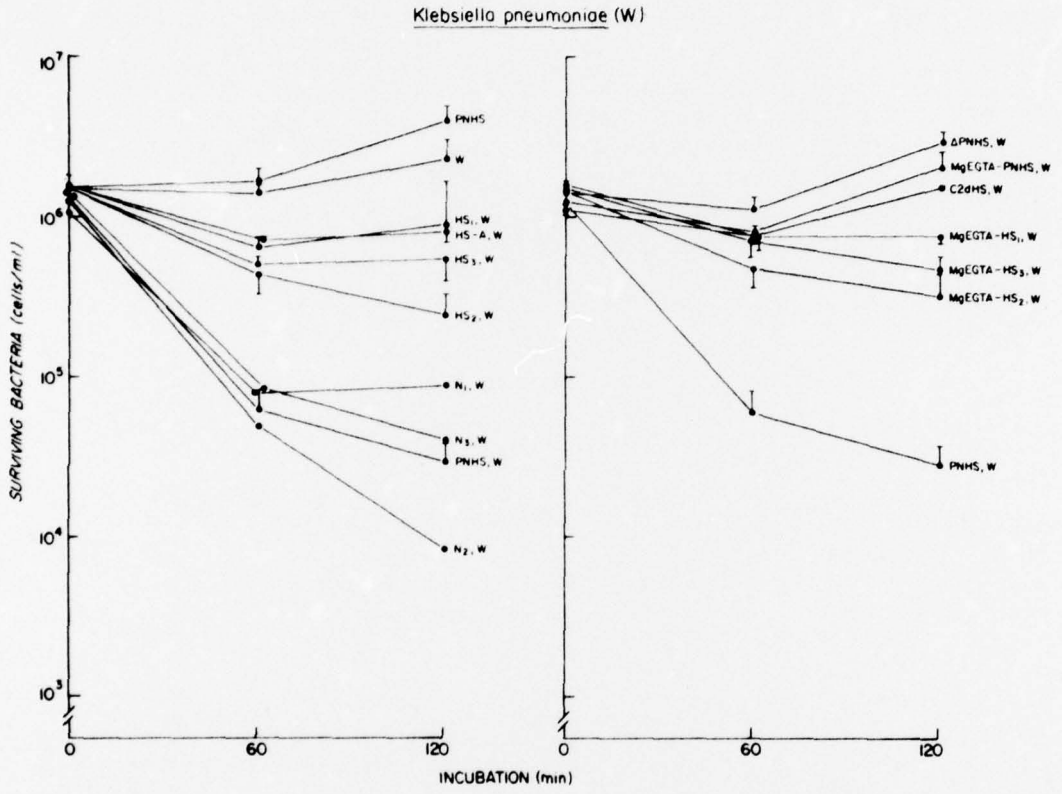


Figure 37. Comparisons of the opsonic activities of normal human sera and sera depleted of immunoglobulin and/or classical pathway activity for K. pneumoniae (W). Abbreviations used were as follows: W, polymorphonuclear leukocytes; PNHS, pooled normal human serum; N_{1-3}, individual normal sera; HS_{1-3}, individual hypogammaglobulinemic sera; HS-A, hypogammaglobulinemic serum further depleted of IgG by immunoadsorption; ΔPNHS, PNHS heated at 56°C for 30 minutes; MgEGTA-PNHS, PNHS treated with 10 mM ethylene glycol tetra-acetic acid (EGTA) and 10 mM $MgCl_2$; C2dHS, C2 deficient human serum; MgEGTA-HS_{1-3}, hypogammaglobulinemic sera treated with 10 mM EGTA and 10 mM $MgCl_2$. The concentration of sera used in the reaction mixtures was 5%. The points represent mean values of 1 to 4 determinations, and each vertical bar represents the standard error of the mean.

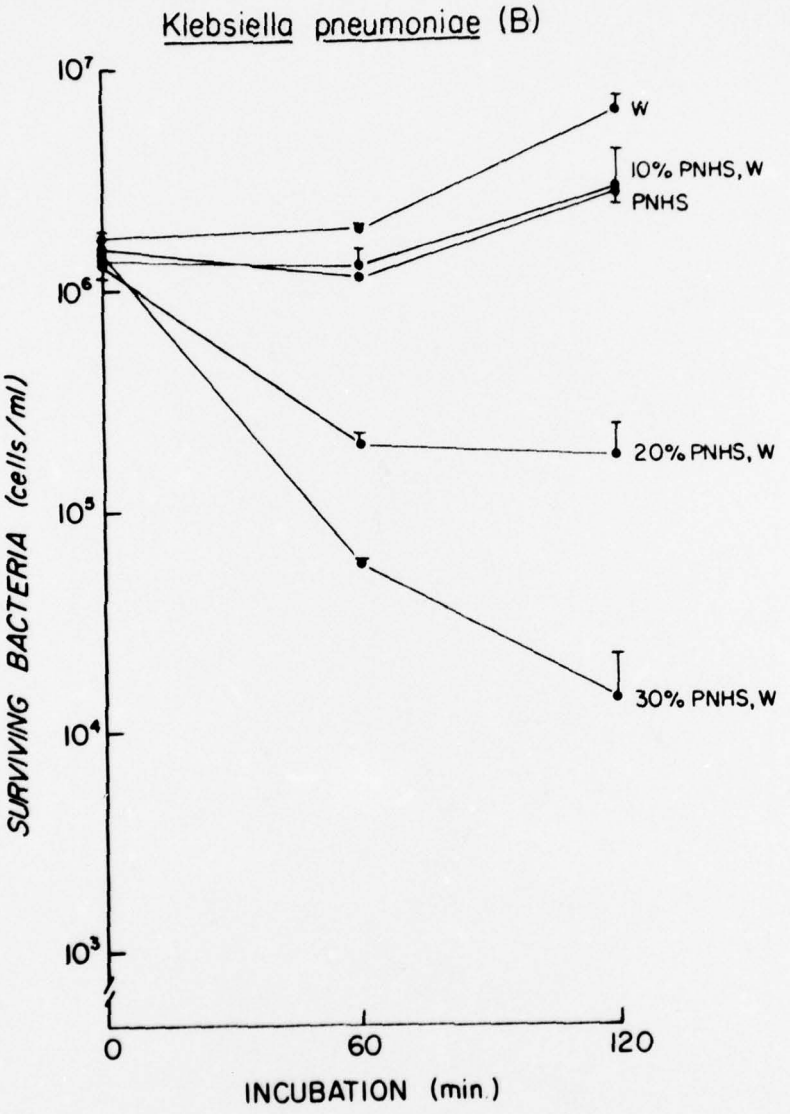


Figure 38. Opsonic activity of increasing concentrations of pooled normal human serum for K. pneumoniae (B). Abbreviations used were as follows: W, polymorphonuclear leukocytes; PNHS, pooled normal human serum. The points represent mean values of 2 to 3 determinations, and each vertical bar represents the standard error of the mean.

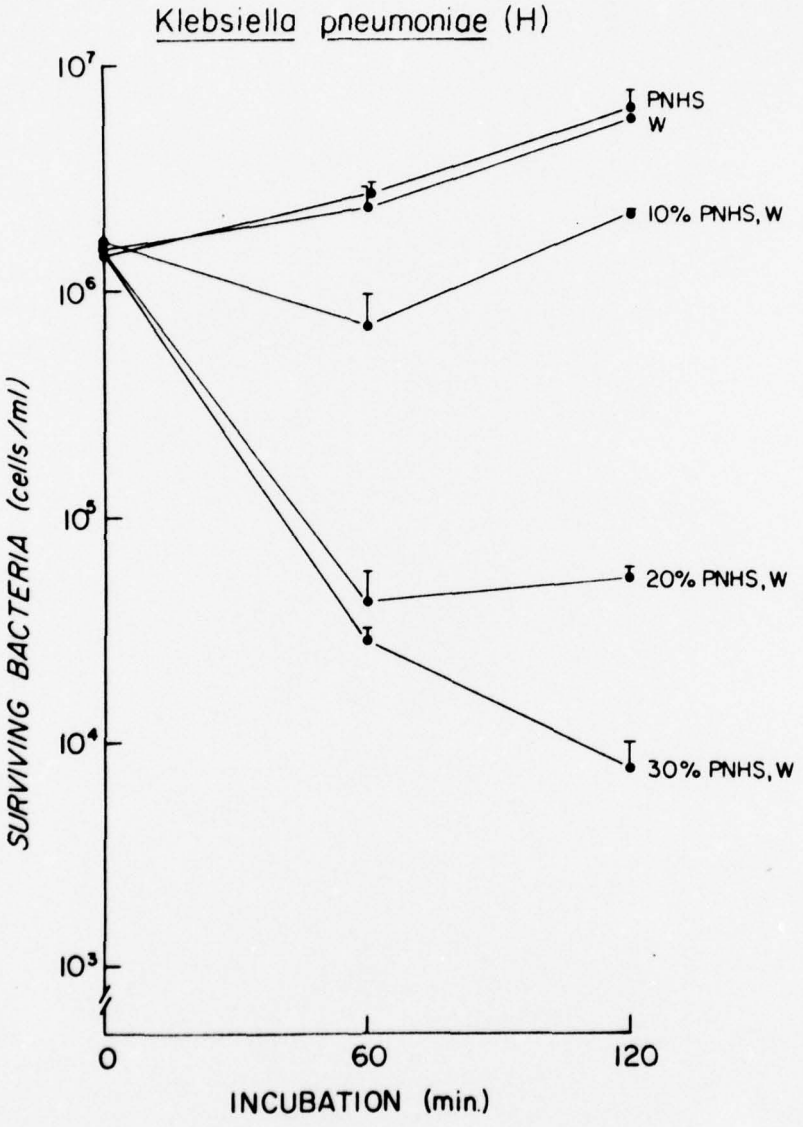


Figure 39. Opsonic activity of increasing concentrations of pooled normal human serum for K. pneumoniae (H). Abbreviations used were as follows: W, polymorphonuclear leukocytes; PNHS, pooled normal human serum. The points represent mean values of 2 determinations, and each vertical bar represents the standard error of the mean.

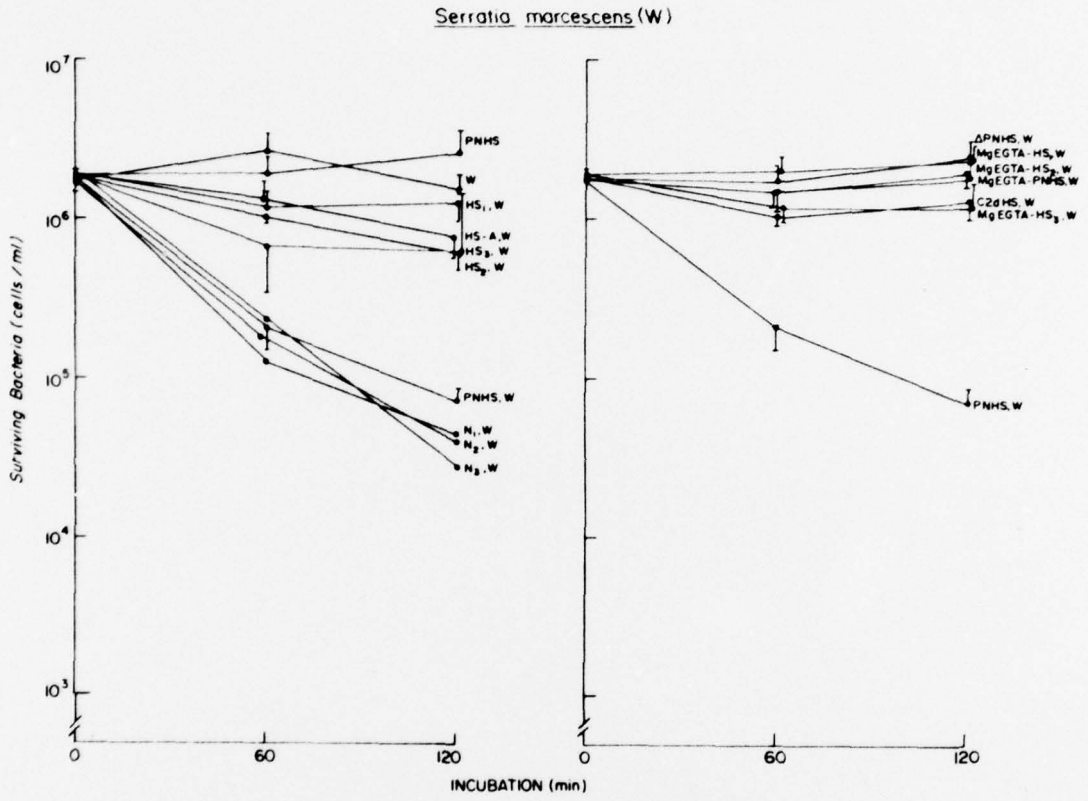


Figure 40. Comparisons of the opsonic activities of normal human sera and sera depleted of immunoglobulin and/or classical pathway activity for S. marcescens (W). Abbreviations used were as follows: W, polymorphonuclear leukocytes; PNHS, pooled normal human serum; N_{1-3}, individual normal sera; HS_{1-3}, individual hypogammaglobulinemic sera; HS-A, hypogammaglobulinemic serum further depleted of IgG by immunoadsorption; ΔPNHS, PNHS heated at 56°C for 30 minutes; MgEGTA-PNHS, PNHS treated with 10 mM ethylene glycol tetra-acetic acid (EGTA) and 10 mM $MgCl_2$; C2dHS, C2 deficient human serum; MgEGTA-HS_{1-3}, hypogammaglobulinemic sera treated with 10 mM EGTA and 10 mM $MgCl_2$. The concentration of sera used in the reaction mixtures was 5%. The points represent mean values of 1 to 4 determinations, and each vertical bar represents the standard error of the mean.

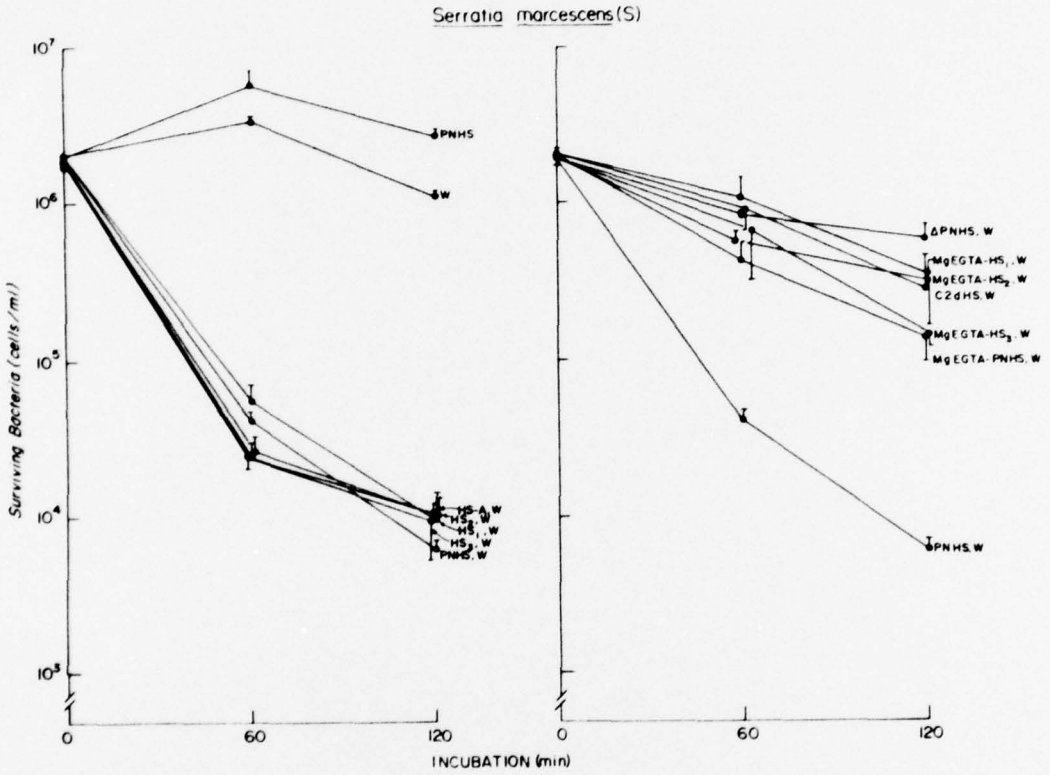


Figure 41. Comparisons of the opsonic activities of normal human serum and sera depleted of immunoglobulin and/or classical pathway activity for S. marcescens (S). Abbreviations used were as follows: W, polymorphonuclear leukocytes; PNHS, pooled normal human serum; HS_{1-3}, individual hypogammaglobulinemic sera; HS-A, hypogammaglobulinemic serum further depleted of IgG by immunoadsorption; ΔPNHS, PNHS heated at 56°C for 30 minutes; MgEGTA-PNHS, PNHS treated with 10 mM ethylene glycol tetra-acetic acid (EGTA) and 10 mM $MgCl_2$; C2dHS, C2 deficient human serum; MgEGTA-HS_{1-3}, hypogammaglobulinemic sera treated with 10 mM EGTA and 10 mM $MgCl_2$. The concentration of sera used in the reaction mixtures was 5%. The points represent mean values of 2 to 4 determinations, and each vertical bar represents the standard error of the mean.

b. Discussion

The requirements for immunoglobulin and the classical and alternative complement pathways for phagocytosis and intracellular killing of the test strains by PMNs are summarized in Table 11. All of the strains were phagocytosed and killed intracellularly only in the presence of serum and PMNs, and not by either alone. In addition, none of the strains was phagocytosed by PMNs in the absence of complement, as evidenced by the inability of decomplemented PNHS to promote phagocytosis. Seven of the thirteen microorganisms included in this study demonstrated a requirement for immunoglobulin for phagocytosis, and utilized only the classical complement pathway {or some other Ca^{++} or C2 dependent pathway, such as the C1 bypass (72)}. Three of the microorganisms required immunoglobulin for phagocytosis and utilized both the classical and alternative pathways. Two of the microorganisms required minimal immunoglobulin, if any, and utilized the classical pathway for phagocytosis. One of the microorganisms did not require immunoglobulin for phagocytosis, and utilized both complement pathways.

It is well known that antibacterial antibodies can trigger the sequential activation of the classical pathway, and this process was probably operative in opsonization of the first group of microorganisms. The role of immunoglobulin in opsonization of those microorganisms which utilized the alternative as well as the classical complement pathway is uncertain. Available evidence indicates that activation of the alternative pathway by inulin or zymosan occurs in the absence of immunoglobulin (19). Lysis of Trypanosoma cyclops (58) and rabbit erythrocytes (59) has also been shown to occur via the alternative pathway without a requirement for conventional antibodies. In contrast, IgG has been shown to participate in lysis of measles virus infected cells via the alternative pathway (60). Additionally, immunoglobulin was shown to be required for bacteriolysis of E. coli via the alternative pathway in bovine serum (73).

It has been hypothesized that the alternative complement pathway may represent a mechanism for the activation of immune defenses when sufficient quantities of antibody are not available for activation of the classical pathway (73). It is interesting that none of the microorganisms tested in our study utilized only the alternative pathway in the absence of immunoglobulin or could be forced to utilize the alternative pathway during phagocytosis in sera depleted of both immunoglobulin and classical pathway activity.

The observation that classical pathway utilization could occur in the absence of immunoglobulin during the opsonic process has not been previously reported. Loos et al. (50) and Morrison and Kline (51) have provided evidence to suggest that the lipid A moiety of lipopolysaccharides can interact directly with the Clq subunit of C1 to initiate classical pathway activation in the absence of immunoglobulin. The results of our study suggest that this mechanism may be operative during opsonization of certain gram-negative aerobic microorganisms.

The minimal concentration of PNHS required to promote maximal phagocytosis and intracellular killing by PMNs was found to vary among the test strains. Eight of the microorganisms required 5% PNHS, and three microorganisms required 10% PNHS for optimal phagocytosis. The other two microorganisms, isolates of

Table 11. Summary of the Requirements for Immunoglobulin and the Alternative and Classical Complement Pathways for Phagocytosis of Clinical Isolates of E. coli, P. mirabilis, K. pneumoniae, and S. marcescens

Microorganism	IgG	Classical Pathway[d]	Alternative Pathway
E. coli (A)[a]	+	+	-
E. coli (F)[a]	+	+	-
E. coli (P)[a]	-	+	-
E. coli (H)[b]	-	+	-
P. mirabilis (H)[a]	+	+	-
P. mirabilis (A)[a]	+	+	+
P. mirabilis (C)[b]	+	+	+
K. pneumoniae (Wo)[a]	+	+	+
K. pneumoniae (W)[b]	+	+	-
K. pneumoniae (B)[a]	+	+	-
K. pneumoniae (H)[a]	+	+	-
S. marcescens (W)[b]	+	+	-
S. marcescens (S)[b]	-	+	+

[a]Microorganisms were isolated from burned patients.

[b]Microorganisms were isolated from medical patients.

[c]Minus sign indicates a minimal or non-existent requirement for immunoglobulin.

[d]Classical pathway or some other Ca^{++} or C2 dependent pathway.

K. pneumoniae, were phagocytosed only when the concentration of PNHS was increased to 20% or 30%. This observation indicated that the level of opsonins required for effective phagocytosis by PMNs varied quantitatively with different strains of bacteria, even within the same species. This interpretation is in agreement with the concept of a quantitative variability of opsonic requirements for optimal phagocytic killing of bacteria, which was recently presented by Guckian et al. (75).

Opsonic requirements for phagocytosis of different strains within the same gram-positive species have been recently investigated. Four S. pneumoniae serotypes were shown to be phagocytosed in hypogammaglobulinemic serum, but to varying degrees of efficiency (76). Similarly, all four serotypes could be phagocytosed to some extent in human serum treated with EGTA and $MgCl_2$ and in C2 deficient serum. A heterogeneity of opsonic requirements among strains of S. aureus has also been reported (52). Two strains were shown to be phagocytosed in hypogammaglobulinemic serum, and were only slightly phagocytosed via the alternative complement pathway. A third strain demonstrated a requirement for immunoglobulin, and efficient phagocytosis proceeded via the alternative pathway. It was proposed that the heterogeneity among S. aureus strains might be related to the presence or absence of the protein A moiety, which could preferentially activate the classical complement pathway via non-specific interaction with the Fc fragment of IgG.

Our data support the observations that a heterogeneity of opsonic requirements exists among different strains of the same species, not only among gram-positive microorganisms as discussed above, but among gram-negative aerobic microorganisms as well. In our study, the test strains isolated from burned patients did not demonstrate a unique pattern of opsonic requirements, in comparison to the same species isolated from medical patients. Rather, a heterogeneity of opsonic requirements for all strains was demonstrated.

Recently, Peterson et al. investigated the resistance of encapsulated S. aureus strains to phagocytosis by PMNs (77). The opsonic requirements between encapsulated and non-encapsulated strains were shown to differ. The presence of a capsule was shown to interfere with opsonization via the classical or alternative complement pathways and with opsonization by heat-stable factors. It was proposed that a capsule might interfere with S. aureus opsonization by covering cell wall peptidoglycan, the portion of the cell wall in which immunological specificity resides. It is interesting to speculate that one of the factors contributing to the differences in opsonic requirements among the test strains in our study might be the presence or absence of capsular material.

One of the most important questions remaining to be answered regards the role of immunoglobulin in the opsonic process. If immunoglobulin is required for effective phagocytosis, as was demonstrated for most of the bacterial strains used in our study, then is it required for activation of the alternative and/or classical complement pathways, or for other steps in the opsonic process (or for a combination of both)? The next section of this report addresses this question by examining the ability of the test strains to activate the classical and alternative pathways in sera containing various immunoglobulin levels.

2. Studies to determine the requirement for immunoglobulin for activation of the alternative complement pathway by gram-negative aerobic microorganisms

a. Results

In the preceding section of this report (C1), the requirements for immunoglobulin and the alternative and classical complement pathways for phagocytosis and intracellular killing of gram-negative aerobic microorganisms were determined. Most of the microorganisms required immunoglobulin and utilized the alternative and/or classical pathway during the opsonic process. Other microorganisms did not require immunoglobulin for phagocytosis and utilized the alternative and/or classical pathway. None of the microorganisms utilized the alternative pathway in the absence of immunoglobulin. The demonstration of a requirement for immunoglobulin for phagocytosis when the alternative complement pathway was utilized led us to investigate the role of immunoglobulin in the opsonic process. The purpose of the present investigation was to determine if immunoglobulin was required for activation of the alternative complement pathway or for other steps in the opsonic process.

The same microorganisms used in our previous study were tested for their ability to convert C3 and to initiate C3 to C9 consumption in human sera depleted of classical complement pathway activity and/or immunoglobulin. Sera were identical to those previously used. Washed heat-killed bacterial cells in saline at a final concentration of 1.0×10^9 cells/ml were incubated in sera for one hour at 37°C. The cells were deposited by centrifugation, and C3 conversion and C3 to C9 consumption were determined. C3 conversion was measured by reduction in the B antigenic determinant of C3 by radial immunodiffusion, and C3 to C9 consumption was measured by a standard hemolytic method using $EAC1\overline{4}$ cells and purified human C2. Saline was substituted for the activating substances in the controls. Statistical analyses were performed by the unpaired Student t test.

Conversion of C3 by the microorganisms in hypogammaglobulinemic serum (HS) was not significantly different from C3 conversion in pooled normal human serum (PNHS), with the exception of E. coli (P) and (H), P. mirabilis (H), K. pneumoniae (W), and S. marcescens (W) and (S) which converted C3 to a lesser extent in HS than in PNHS (Table 12). C3 conversion by all of the microorganisms in HS further depleted of IgG by immunoadsorption (HS-A) was significantly decreased, in comparison to C3 conversion in PNHS. In contrast, inulin converted C3 equivalently in PNHS, HS, and HS-A. All of the microorganisms initiated C3 to C9 consumption in HS equivalent to, or slightly higher than in PNHS; the microorganisms initiated C3 to C9 consumption in HS-A to a lesser extent than in PNHS (Table 13). These results indicated that immunoglobulin was required for optimal complement activation by the microorganisms. In contrast, immunoglobulin was not required for complement activation by inulin.

The ability of the microorganisms to convert C3 in sera depleted of immunoglobulin and/or classical pathway activity was next investigated. All of the microorganisms converted C3 in PNHS treated with 10 mM ethylene glycol tetraacetic acid (EGTA) and 10 mM $MgCl_2$ (MgEGTA-PNHS) equivalent to that in untreated PNHS, with the exception of E. coli (A), (P), and (H), and S. marcescens (S) which demonstrated slightly lower C3 conversion in MgEGTA-PNHS (Table 14). No

Table 12. C3 Conversion by Heat-Killed Gram-Negative Bacteria in Pooled Normal Human Serum (PNHS), Hypogammaglobulinemic Serum (HS), and HS Further Depleted of IgG by Immunoadsorption (HS-A)

Activating[a] Substance	C3 Conversion (%)[c]		
	PNHS	HS	HS-A
Inulin[b]	59.4	53.3	56.2
E. coli (A)[d]	46.7	38.8	21.3
E. coli (F)[d]	46.0	40.8	19.8
E. coli (P)[d,e]	57.8	46.0	36.7
E. coli (H)[d,e]	52.8	42.0	11.5
P. mirabilis (H)[d,e]	50.3	36.5	17.0
P. mirabilis (A)[d]	46.0	39.3	12.5
P. mirabilis (C)[d]	50.3	41.5	13.5
K. pneumoniae (Wo)[d]	52.0	48.3	29.8
K. pneumoniae (W)[d,e]	59.3	49.3	29.7
K. pneumoniae (B)[d]	45.0	45.3	25.0
S. marcescens (W)[d,e]	70.3	55.3	55.3
S. marcescens (S)[d,e]	64.8	41.0	33.2

[a]Final concentration of bacteria was 1.0×10^9 cells/ml.

[b]Final concentration of inulin was 10 mg/ml.

[c]Mean values of 2 to 6 determinations are presented.

[d]PNHS vs. HS-A; $p = < 0.05$.

[e]PNHS vs. HS; $p = < 0.05$.

Table 13. C3 to C9 Consumption by Heat-Killed Gram-Negative Bacteria in Pooled Normal Human Serum (PNHS), Hypogammaglobulinemic Serum (HS), and HS Further Depleted of IgG by Immunoadsorption (HS-A)

Activating[a] Substance	C3 to C9 Consumption (%)[c]		
	PNHS	HS	HS-A
Inulin[b]	83	92	90
E. coli (A)	72	92	52
E. coli (F)	60	92	57
E. coli (P)	91	93	65
E. coli (H)	68	92	9.5
P. mirabilis (H)	71	92	10
P. mirabilis (A)	92	92	20
P. mirabilis (C)	92	92	10
K. pneumoniae (Wo)	92	92	71
K. pneumoniae (W)	92	92	62
K. pneumoniae (B)	72	92	48
S. marcescens (W)	92	92	90
S. marcescens (S)	91	93	90

[a]Final concentration of bacteria was 1.0×10^9 cells/ml.

[b]Final concentration of inulin was 10 mg/ml.

[c]Values from one determination are presented.

Table 14. C3 Conversion by Heat-Killed Gram-Negative Bacteria in Sera Depleted of Classical Pathway Activity and/or Immunoglobulin

Activating[a] Substance	C3 Conversion (%)[c]					
	PNHS[d]	MgEGTA-PNHS[e]	HS[f]	MgEGTA-HS[g]	HS-A[h]	MgEGTA-HS-A[i]
Inulin[b]	59.4	60.0	53.3	52.0	53.3	52.7
E. coli (A)[j]	46.7	35.3	38.8	41.8	18.0	19.0
E. coli (F)	46.0	36.0	40.8	44.7	16.0	17.0
E. coli (P)[j]	57.8	46.7	46.0	43.0	32.3	24.6
E. coli (H)[j]	52.8	42.0	42.0	39.5	17.3	21.3
P. mirabilis (H)	50.3	40.7	36.5	38.5	23.0	24.5
P. mirabilis (A)	46.0	38.0	39.3	38.8	24.0	22.5
P. mirabilis (C)	50.3	44.3	41.5	39.5	32.0	24.0
K. pneumoniae (Wo)	52.0	46.0	48.3	42.3	24.0	13.0
K. pneumoniae (W)	59.3	55.0	49.3	46.5	25.0	20.5
K. pneumoniae (B)	45.0	44.3	45.3	39.5	19.0	26.5
S. marcescens (W)	70.3	70.7	55.3	49.8	31.3	34.7
S. marcescens (S)[j]	64.8	54.3	41.0	43.8	29.7	27.0

[a]Final concentration of bacteria was 1.0×10^9 cells/ml.

[b]Final concentration of inulin was 10 mg/ml.

[c]Mean values of 2 to 6 determinations are presented.

[d]PNHS = pooled normal human serum.

[e]MgEGTA-PNHS = PNHS treated with 10 mM EGTA and 10 mM $MgCl_2$.

[f]HS = hypogammaglobulinemic serum.

[g]MgEGTA-HS = HS treated with 10 mM EGTA and 10 mM $MgCl_2$.

[h]HS-A = HS further depleted of IgG by immunoadsorption.

[i]MgEGTA-HS-A = HS-A treated with 10 mM EGTA and 10 mM $MgCl_2$.

[j]PNHS vs. MgEGTA-PNHS; $p = < 0.05$.

significant differences in C3 conversion by the microorganisms were demonstrated in HS and HS treated with 10 mM EGTA and 10 mM $MgCl_2$ (MgEGTA-HS), or in HS-A and HS-A treated with 10 mM EGTA and 10 mM $MgCl_2$ (MgEGTA-HS-A). Inulin converted C3 equivalently in all treated and untreated sera. No differences in C3 to C9 consumption by the microorganisms or inulin were demonstrated in PNHS and MgEGTA-PNHS, or in HS and MgEGTA-HS (Table 15). These results indicated that all of the microorganisms were capable of efficiently activating complement via the alternative pathway, and that the requirement for immunoglobulin was minimal or non-existent for alternative complement pathway activation.

b. Discussion

Available evidence regarding the immunoglobulin requirement for alternative pathway activation has indicated that the requirement differs according to the activating substance. Activation of the alternative complement pathway by inulin or zymosan appears to occur in the absence of immunoglobulin (19). Lysis of Trypanosoma cyclops (58) and rabbit erythrocytes (59) via the alternative pathway has been shown to occur without a requirement for antibodies. In contrast, immunoglobulin has been shown to participate in lysis of measles virus infected cells via the alternative pathway (60). In addition, immunoglobulin was shown to be required for bacteriolysis of E. coli via the alternative pathway in bovine serum (73). The results of our investigation indicated that optimal complement activation by the gram-negative microorganisms required immunoglobulin, suggesting that classical pathway activation was involved. However, immunoglobulin was not shown to be required for alternative pathway activation by the microorganisms.

Although all of the microorganisms used in our study were shown to be capable of activating the alternative complement pathway when added directly to human sera, only four of the 13 microorganisms utilized the alternative pathway during phagocytosis. One explanation for this observation is that the opsonic products derived via alternative pathway activation were less efficient in promoting phagocytosis, in comparison to products derived via classical pathway activation. Another hypothesis is suggested by the recently published data of Fearon and Austen (67). These investigators demonstrated that zymosan and rabbit erythrocytes provided privileged sites that protected C3b deposited on their surfaces during alternative pathway activation from the action of the regulatory proteins C3b inactivator (C3b INA) and β1H. Zymosan and rabbit erythrocytes thereby allowed transition from low grade C3 cleavage to the amplification stage of C3 cleavage during alternative pathway activation. If this mechanism was involved in alternative pathway activation by the microorganisms, protective sites on the microbial surface might become altered during the phagocytic event. If this happened, utilization of the alternative pathway during phagocytosis of the microorganisms would not be demonstrated.

Our results also suggested that immunoglobulin was not required during phagocytosis for altering the microbial surface to protect C3b deposited by alternative pathway activation from the action of C3b INA and β1H. Immunoglobulin was not shown to be required for alternative pathway activation by the bacteria, and therefore must be required for other steps in the opsonic process. For example, immunoglobulin may be required for phagocytic uptake of the microorganisms. Mantovani (78) and more recently, Ehlenberger and Nussenzweig (79)

Table 15. C3 to C9 Consumption by Heat-Killed Gram-Negative Bacteria in Sera Depleted of Classical Pathway Activity and/or Immunoglobulin

Activating[a] Substance	C3 to C9 Consumption (%)[c]			
	PNHS[d]	MgEGTA-PNHS[e]	HS[f]	MgEGTA HS[g]
Inulin[b]	83	81	92	91
E. coli (A)	72	73	92	91
E. coli (F)	60	63	92	91
E. coli (P)	91	81	93	90
E. coli (H)	68	70	92	91
P. mirabilis (H)	71	69	92	91
P. mirabilis (A)	92	92	92	91
P. mirabilis (C)	92	85	92	87
K. pneumoniae (Wo)	92	93	92	91
K. pneumoniae (W)	92	90	92	91
K. pneumoniae (B)	72	90	92	91
S. marcescens (W)	92	92	92	91
S. marcescens (S)	91	94	93	90

[a]Final concentration of bacteria was 1.0×10^9 cells/ml.

[b]Final concentration of inulin was 10 mg/ml.

[c]Values from one determination are presented.

[d]PNHS = pooled normal human serum.

[e]MgEGTA-PNHS = PNHS treated with 10 mM EGTA and 10 mM $MgCl_2$.

[f]HS = hypogammaglobulinemic serum.

[g]MgEGTA-HS = HS treated with 10 mM EGTA and 10 mM $MgCl_2$.

have demonstrated separate but synergistic roles for IgG and C3b in erythrophagocytosis; IgG was required for ingestion of erythrocytes by polymorphonuclear leukocytes, whereas C3b was required for attachment. It has also been demonstrated that PMNs increased their oxidative metabolism upon binding to C3-coated Sepharose beads, without requiring the presence of IgG (80). However, degranulation and release of lysosomal enzymes occurred only when the PMNs encountered C3 in the presence of IgG. It could perhaps be speculated that IgG might facilitate lysosomal fusion and the subsequent release of digestive enzymes into the phagolysosome, thereby increasing intracellular killing of the microorganisms.

3. Studies to determine the mechanisms of complement activated by lipopolysaccharide and lipid A prepared from gram-negative aerobic bacilli

a. Results

In our previous studies, intact cells of gram-negative aerobic bacilli were shown to be capable of activating the alternative complement pathway (53). Efficient alternative pathway activation was achieved utilizing washed heat-killed cells of Escherichia coli 075, Proteus mirabilis 7056, Pseudomonas aeruginosa 73044, and Salmonella minnesota S form (SF1114). S. minnesota chemotype Rb which lacks the O antigen and acetylglucosamine attached to the terminal glucose but contains the rest of the basal core and S. minnesota chemotype Re which contains only KDO and lipid A were not found to be as efficient as the other gram-negative bacilli in activating the alternative pathway. These microorganisms were, however, highly active in initiating classical pathway activation. These results provided preliminary evidence to suggest that the polysaccharide moiety of the lipopolysaccharide (LPS) activated the alternative complement pathway, and the lipid A moiety activated the classical pathway. The purpose of the present investigation was to determine if our results concerning the moieties of the bacterial cell wall involved in activation of the classical and alternative pathways could be confirmed utilizing purified lipid A and LPS prepared from our test strains.

LPS and lipid A were prepared from E. coli 075, P. mirabilis 7056, P. aeruginosa 73044, S. minnesota SF1114, and E. coli (A), a strain isolated from the blood culture of a burned patient. Since there is evidence that the method of purification of the cell wall components may influence their biological activities (49,50). LPS was prepared by phenol-water extraction and tested before and after RNAse treatment. In addition, lipid A was prepared by hydrolysis with two different acids. LPS was extracted by the phenol water method of Westphal and Jann (81). The water extracts were precipitated by 2.5 volumes of ethanol, and the precipitate was deposited by centrifugation after 18 hours at 4°C. The preparation was dissolved in water and lyophilized; this preparation was designated LPS-1. LPS-1 was further purified by treatment with RNAse (82). Twenty mg per ml of LPS-1 in 0.01 M phosphate buffered saline, pH 7.0, was incubated for 1 hour at room temperature with RNAse (final concentration of 25 µg/ml). This solution was dialyzed extensively at 4°C against the same buffer and lyophilized. This preparation was designated LPS-2. LPS-1 and LPS-2 preparations were redissolved in 0.01 M phosphate buffered saline, pH 7.0, prior to use. Lipid A-1 was prepared from LPS-1 after hydrolysis in 0.05 N HCl at 100°C for 1 hour (82). Lipid A-2 was prepared from LPS-1 after hydrolysis in 1%

glacial acetic acid at 100°C for 2 hours (51). All lipid A preparations were washed twice with distilled water, solubilized with 1% triethylamine in phosphate buffered saline, and sonicated prior to use (83).

Increasing concentrations of LPS and lipid A prepared from the microorganisms were added to pooled normal human serum (PNHS) and normal guinea pig serum (NGPS), and C3 to C9 consumption was measured (84). Controls for the experiments were PNHS or NGPS treated with phosphate buffered saline or phosphate buffered saline containing 1% triethylamine. No consumption of C3 to C9 was achieved until the preparations were added at final concentrations of 1 mg/ml or greater. Therefore, in the experiments to be described, the preparations were used at final concentrations of 1 mg/ml and 5 mg/ml respectively.

Washed heat-killed bacterial cells, LPS, and lipid A prepared from the microorganisms were compared for their ability to initiate C3 to C9 consumption in untreated PNHS and in PNHS treated with 10 mM ethylene glycol tetra-acetic acid (EGTA) and 10 mM $MgCl_2$ (MgEGTA-PNHS). Treatment of PNHS with EGTA and magnesium ions was used to block classical pathway activity as described in sections C1 and C2 of this report. LPS-1 prepared from all of the microorganisms was as efficient as heat-killed (Δ) cells in initiating C3 to C9 consumption in untreated PNHS (Table 16). LPS-2 was less efficient than LPS-1 in activating C3 to C9 in PNHS. No consistent differences were observed between the abilities of lipid A-1 and lipid A-2 to initiate C3 to C9 consumption in PNHS, although all preparations were less efficient than LPS-1. C3 to C9 consumption by all of the activating substances was reduced in MgEGTA-PNHS. Δ cells and LPS-1 preparations were the most efficient activating substances in initiating C3 to C9 consumption in MgEGTA-PNHS.

Similar results were obtained when C3 to C9 consumption by the activating substances in NGPS and C4 deficient guinea pig serum (C4dGPS) was determined. With the exceptions of E. coli (A) and P. mirabilis 7056, LPS-1 preparations were as efficient or more efficient than Δ cells in initiating C3 to C9 consumption in NGPS (Table 17). LPS-2 preparations were less efficient than LPS-1 preparations in initiating C3 to C9 consumption in NGPS. LPS 2 prepared from P. mirabilis 7056 and S. minnesota SF 1114 initiated C3 to C9 consumption in NGPS only at concentrations of 5 mg/ml, and LPS-2 prepared from E. coli (A) had no anticomplementary activity at this concentration. No consistent differences between lipid A-1 and lipid A-2 preparations were observed, however all lipid A preparations were equally or less efficient than LPS-1 preparations in initiating C3 to C9 consumption in NGPS. C3 to C9 consumption by all of the activating substances was markedly reduced in C4dGPS. Δ cells of E. coli A and P. mirabilis 7056 were the only activating substances that efficiently initiated consumption of C3 to C9 in C4dGPS (> 31%). C3 to C9 consumption by inulin and cobra venom factor was 55% and 77% respectively, indicating that the alternative complement pathway was intact in C4dGPS. In addition, purified guinea pig C4 was shown to restore lysis of sensitized erythrocytes in C4dGPS, indicating that only C4 was deficient in the serum.

b. Discussion

The LPS cell wall component of gram-negative aerobic bacilli has been repeatedly shown to be anticomplementary (46-51,85-90). Evidence has been

Table 16. Consumption of C3 to C9 by Heat-Killed Bacteria, Lipopolysaccharide (LPS) and Lipid A in PNHS[a] and MgEGTA-PNHS[b]

Microorganism	Preparation Tested	C3 to C9 Consumption (%)[c]			
		PNHS		MgEGTA-PNHS	
		1 mg/ml[d]	5 mg/ml[d]	1 mg/ml[d]	5 mg/ml[d]
E. coli 075	Heat-killed (Δ) cells[e]	74		64	
	LPS-1[f,g]	93	92	24	81
	LPS-2[g,h]	57	40	16	32
	Lipid A-1[i,j]	32	40	16	8
	Lipid A-2[j,k]	30	30	4	8
E. coli (A)	Δ cells	94		84	
	LPS-1	94	93	24	81
	LPS-2	49	39	12	19
	Lipid A-1	45	41	32	38
	Lipid A-2	52	43	8	23
P. aeruginosa 73044	Δ cells	97		80	
	LPS-1	97	65	21	51
	LPS-2	57	62	26	38
	Lipid A-1	46	48	4	8
	Lipid A-2	38	48	0	0
P. mirabilis 7056	Δ cells	92		84	
	LPS-1	46	92	16	77
	LPS-2	32	66	12	44
	Lipid A-1	32	41	8	16
	Lipid A-2	34	68	46	39
S. minnesota SF1114	Δ cells	94		84	
	LPS-1	94	93	26	81
	LPS-2	53	39	16	9
	Lipid A-1	55	48	0	12
	Lipid A-2	48	21	0	0

[a]PNHS = pooled normal human serum.

[b]MgEGTA-PNHS = pooled normal human serum treated with 10 mM EGTA and 10 mM $MgCl_2$.

[c]Values of single determinations are presented.

[d]Final concentrations of LPS and lipid A tested in the assays.

[e]Washed bacterial cells heat-killed at 70°C for 1 hour were tested at a final concentration of 1.0×10^9 cells/ml.

[f]LPS-1 was phenol-water extracted ethanol precipitated LPS prior to RNAse treatment.

[g]C3 to C9 consumption was calculated using phosphate buffered saline treated PNHS or MgEGTA-PNHS as the control.

[h]LPS-2 was phenol-water extracted ethanol precipitated LPS after RNAse treatment.

[i]Lipid A-1 was prepared by acid hydrolysis with HCl.

[j]C3 to C9 consumption was calculated using PNHS or MgEGTA-PNHS treated with phosphate buffered saline containing 1% triethylamine.

[k]Lipid A-2 was prepared by acid hydrolysis with glacial acetic acid.

Table 17. Consumption of C3 to C9 by Heat-Killed Bacteria, Lipopolysaccharide (LPS) and Lipid A in NGPS[a] and C4dGPS[b]

Microorganism	Preparation Tested	C3 to C9 Consumption (%)[c]			
		NGPS		C4dGPS	
		1 mg/ml[d]	5 mg/ml[d]	1 mg/ml[d]	5 mg/ml[d]
E. coli 075	Heat-Killed (Δ) cells[e]	80		17	
	LPS-1[f,g]	88	96	5.7	4.9
	LPS-2[g,h]	68	68	1.4	0
	Lipid A-1[i,j]	67	83	0	0
	Lipid A-2[j,k]	66	68	7.5	0
E. coli (A)	Δ cells	91		59	
	LPS-1	51	46	3.1	9.2
	LPS-2	0	0	1.5	6.2
	Lipid A-1	68	65	6.1	3.8
	Lipid A-2	56	68	2.3	5.9
P. aeruginosa 73044	Δ cells	87		5.8	
	LPS-1	78	91	0	0
	LPS-2	67	68	0	0
	Lipid A-1	68	64	13.8	7.3
	Lipid A-2	40	37	4.9	8.1
P. mirabilis 7056	Δ cells	91		32	
	LPS-1	44	83	5	2
	LPS-2	0	40	0	0
	Lipid A-1	69	75	13.5	21.1
	Lipid A-2	81	88	25.5	25.4
S. minnesota SF1114	Δ cells	75		9.1	
	LPS-1	96	99	0	2.9
	LPS-2	0	44	0	2
	Lipid A-1	77	73	0	0
	Lipid A-2	75	67	0	0

[a]NGPS = normal guinea pig serum.

[b]C4dGPS = C4 deficient guinea pig serum.

[c]Values of single determinations are presented.

[d]Final concentrations of LPS and lipid A tested in the assays.

[e]Washed bacterial cells heat-killed at 70°C for 1 hour were tested at a final concentration of 1.0×10^9 cells/ml.

[f]LPS-1 was phenol-water extracted ethanol precipitated LPS prior to RNAse treatment.

[g]C3 to C9 consumption was calculated using NGPS or C4dGPS treated with phosphate buffered saline.

[h]LPS-2 was phenol-water extracted ethanol precipitated LPS after RNAse treatment.

[i]Lipid A-1 was prepared by acid hydrolysis with HCl.

[j]C3 to C9 consumption was calculated using NGPS or C4dGPS treated with phosphate buffered saline containing 1% triethylamine.

[k]Lipid A-2 was prepared by acid hydrolysis with glacial acetic acid.

provided to support the concept that LPS is capable of activating complement by a C142-independent mechanism (47,48,86-88). More recently, several investigators have shown that LPS activates the classical as well as the alternative complement pathway (49,51,89,90). The primary objective of several of these investigations was to determine the moiety of the LPS which was responsible for its anticomplementary activity (49,51). Galanos et al. (49) demonstrated that LPS prepared from Salmonella and E. coli S and R mutant strains by extraction with phenol-water or phenol-chloroform-petroleum ether varied in their ability to reduce total hemolytic complement in normal guinea pig serum. Lipid A prepared by glacial acetic acid hydrolysis from both active and non-active LPS preparations was, however, strongly anticomplementary when made water-soluble by the aid of carriers; however, lipid A solubilized by triethylamine or pyridine had no anticomplementary activity. Morrison and Kline (51) showed that lipid A prepared from S. typhimurium R60 LPS by glacial acetic acid hydrolysis and solubilized in triethylamine and LPS prepared from S. minnesota chemotype Re were more efficient on a weight basis in reducing total hemolytic complement in normal human sera than commercially prepared or phenol-water extracted LPS from smooth strains of E. coli. Consumption of early components (C1, 4, and 2) by S. minnesota chemotype Re LPS was greater than by the E. coli LPS preparations. The capacity of S. minnesota chemotype Re LPS to convert C3 in PNHS was completed abbrogated in C2 deficient human serum, whereas C3 conversion by E. coli LPS in PNHS and C2 deficient serum was equivalent. The investigators concluded from these observations that the ability of LPS to activate the classical pathway was a function of the lipid A moiety, and that the polysaccharide region was responsible for alternative pathway activation. In addition to these investigations, Loos et al. showed that lipid A interacts directly with the C1q subunit of C1 to inhibit its ability to form C3 convertase activity with purified C4 and C2 (50).

In the studies which have been described above (46-51,81-90). LPS was prepared from the bacterial strains by phenol-water extraction or phenol-chloroform-petroleum ether extraction, and RNA was not removed from the preparations. Leive et al. have shown that phenol-water extracted LPS contains approximately 50% contaminating RNA (82). Purification of LPS by fractionation on Sepharose 4B, as was performed in the experiments of Morrison and Kline (51), would only remove RNA which was not complexed to LPS. In our experiments, RNA appeared to play a definite role in the anticomplementary activity of LPS, because removal of RNA by treatment of LPS with RNAse either markedly reduced or completely abolished the ability of the LPS preparation to initiate C3 to C9 consumption. In addition, lipid A prepared by hydrolysis with two different acids and solubilized in triethylamine was not found to be more efficient on a weight basis in activating complement than phenol-water extracted LPS prior to RNAse treatment. Lipid A was as efficient on a weight basis as phenol-water extracted RNAse treated LPS in initiating C3 to C9 consumption in normal human serum, and was only slightly more efficient in activating terminal components in normal guinea pig serum.

Phenol-water extracted LPS after removal of contaminating RNA was markedly less efficient than heat-killed intact cells in activating terminal complement components in either human or guinea pig serum. This observation suggested that the ability of the heat-killed bacterial cells to activate complement was not solely dependent on the LPS or lipid A in the bacterial cell wall, or that the

procedures for removal of LPS and lipid A from the bacterial cells altered their anticomplementary activities.

Phenol-water extracted LPS preparations before or after RNAse treatment were more efficient than lipid A preparations in activating the alternative complement pathway in human serum. However, all of the preparations activated terminal components in normal human serum much more efficiently than in human serum lacking classical pathway activity. These results suggested that lipid A could only activate the classical or another calcium dependent pathway in human serum, and that LPS activated the classical as well as the alternative pathway. However utilizing C4 deficient guinea pig serum, neither LPS nor lipid A preparations consumed terminal complement components, suggesting that lipid A and LPS were incapable of activating the alternative pathway in this serum. The results could be explained by the lack of antibody to LPS or lipid A in the C4 deficient guinea pig serum, since intact heat-killed bacterial cells also did not activate terminal components in this serum as efficiently as in normal guinea pig serum. This concept is further supported by the observation that intact cells activated the alternative pathway in pooled normal human serum which contained naturally occurring anti-bacterial antibodies.

In summary, the results of our investigation indicated that purified lipid A and LPS activated terminal complement components in normal human or guinea pig sera equally efficiently, and that there were other heat-stable components on the bacterial cell surface with anticomplementary activity. LPS appeared to be capable of activating the alternative and classical pathways, whereas lipid A appeared to be capable of efficiently activating only the classical pathway. This finding is in agreement with the observations of Morrison and Kline who showed that LPS converted C3 in C2 deficient human serum, but that lipid A did not (51). In addition, the results of the present investigation support our earlier observations using intact heat-killed cells of S. minnesota mutant strains which showed that chemotype S was a more efficient activator of the alternative pathway than chemotypes Rb or Re (53). Our future studies will be focused on substantiating the preliminary observations in this investigation and on determining the requirement for immunoglobulin for consumption of the classical and/or alternative pathways by purified LPS and lipid A.

4. In vitro interaction of human polymorphonuclear leukocytes and serum with strains of Candida albicans

a. Results

In our previous studies, strains of Candida albicans isolated from burned patients were shown to be resistant to the bactericidal activity of human polymorphonuclear leukocytes (PMNs), even in the presence of excessive concentrations of normal human serum (Annual Summary Report, June, 1977). The present investigation was undertaken to determine if strains of C. albicans isolated from burned patients were unique in terms of their inability to be killed intracellularly by PMNs.

The interaction of human PMNs and pooled normal human serum (PNHS) with C. albicans strains isolated from burned patients, medical patients, and other sources was compared. Strains isolated from burned patients were obtained from

the Shriners Burn Institute, Cincinnati, O., and were designated CA-2, CA-13, CA-14, CA-15, CA-S, CA-F, CA-Ke, and CA-Bo. The strains cited above were isolated from blood (CA-F, CA-Ke) or from wound cultures (CA-2, CA-13, CA-14, CA-15, CA-S, and CA-Bo). C. albicans strains isolated from medical patients were obtained from the Central Bacteriology Laboratory of the Cincinnati General Hospital, Cincinnati, O. Two of the strains were isolated from blood cultures (CA-W, CA-Ba), and the other strain was isolated from cerebral spinal fluid (CA-Kn). Two additional C. albicans strains were purchased from the American Type Culture Collection (Rockville, Md.), CA-ATCC 28366 (human mouth isolate) and CA-ATCC 28121 (stool specimen isolate). Medical patient isolates, ATCC isolates, and CA-S, CA-F, CA-Ke, and CA-Bo were immediately transferred to Sabouraud 2% dextrose broth, incubated at 37°C overnight, and frozen in small aliquots at -70°C. CA-2, CA-13, CA-14, and CA-15 had been repeatedly subcultured prior to being obtained, and after receipt were handled as described for the other isolates. The frozen cultures were thawed, and 0.2 ml of each was inoculated into a tube containing 5 ml of Sabouraud 2% dextrose broth and incubated at 37°C overnight.

Reaction mixtures consisted of 5.0×10^6 PMNs, 1.0×10^6 C. albicans, and PNHS (10%, 20%, 30%, 40%) in a final volume of 1 ml of Hank's balanced salt solution (HBSS). HBSS was substituted for the PMNs, PNHS, or both in the controls. Reaction mixtures and controls were rotated end over end for 120 minutes at 37°C. Aliquots were diluted in distilled water at 0 time, 30, 60, and 120 minutes and plated on Sabouraud 2% dextrose agar. The plates were incubated at 37°C overnight, and the colonies were enumerated. The results are expressed in surviving colony forming units (cfu) per ml.

For two of the strains of C. albicans isolated directly from burned patients (CA-S and CA-F), killing was maximal by PNHS in the absence of PMNs (Figure 42). For the other two strains (CA-Bo and CA-Ke), killing was maximal by PMNs alone in the absence of PNHS. Killing of the C. albicans strains isolated from burned patients and subcultured repeatedly in the laboratory was maximal in the absence of serum or PMNs (CA-2, CA-14, and CA-15) or by PMNs alone (CA-13) (Figure 43). For two of the three strains of C. albicans isolated from medical patients, killing was maximal in the absence of PMNs or PNHS (CA-Kn and CA-Ba) or in the presence of PMNs alone (CA-W) (Figure 44). Killing of CA-W was not substantially improved by the addition of PNHS. Reduction in counts of all of the strains described above, except those killed by PNHS alone, was under one log. Addition of PNHS enhanced killing of the ATCC isolates by PMNs (Figure 45). Maximal reduction in counts of C. albicans strains obtained from the ATCC was approximately one log. In addition, direct killing of the strains by PNHS was equivalent to killing of strains in the presence of HBSS alone.

b. Discussion

C. albicans infections are commonly observed in burned patients and other compromised hosts, constituting a significant mortality in this population (91-96). Within invasive lesions, pseudohyphal and hyphal forms of Candida are more prominent than yeast forms (97) and must be cleared if the host is to survive. Pseudohyphal and hyphal forms of Candida can be cleared in at least some systemic infections in experimental animals (98) and in man (91-96,99,100). Reproducible quantitative determinations of viable hyphae and pseudohyphae have been difficult. Most in vitro studies have concentrated on the ability of leukocytes to ingest and kill yeast forms. Such studies (101-105) as well as the observed clinical association of neutropenia with disseminated

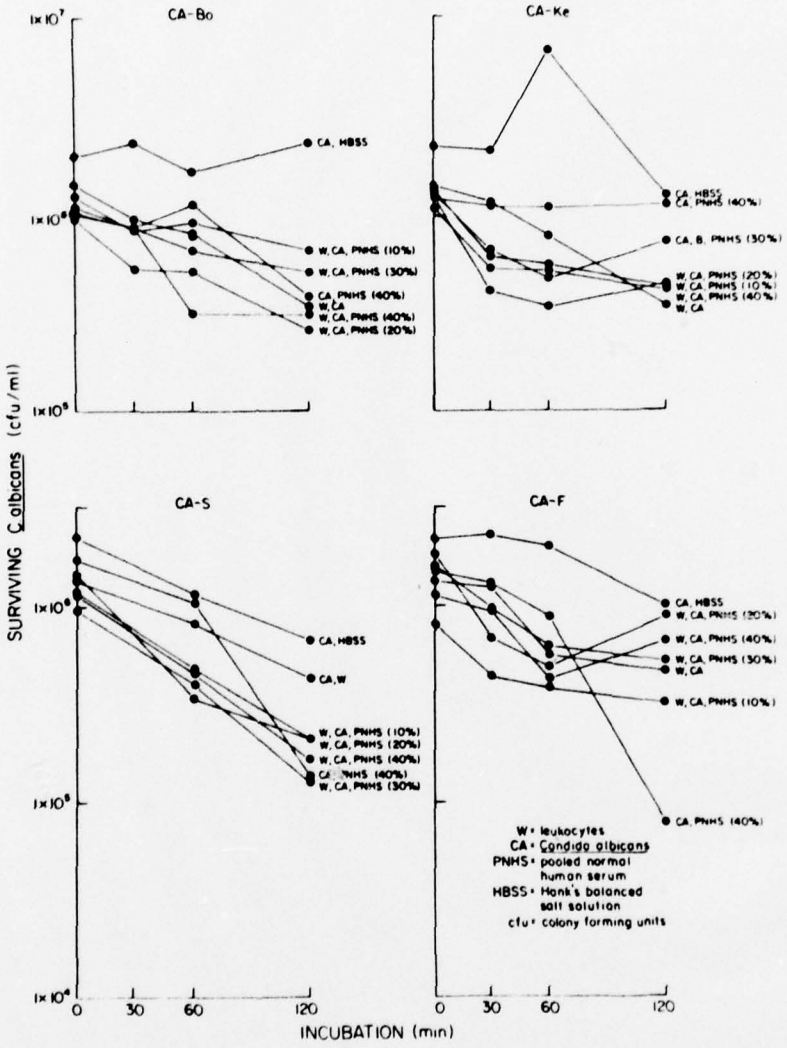


Figure 42. Interaction of pooled normal human serum (PNHS) and PMNs with strains of C. albicans isolated directly from burned patients. The following reaction mixtures were tested: 1) PMNs (W), C. albicans (CA); 2) W, CA, PNHS (10%, 20%, 30%, 40%); 3) CA, PNHS (40%); and 4) CA, Hank's balanced salt solution (HBSS). The points represent values of single determinations.

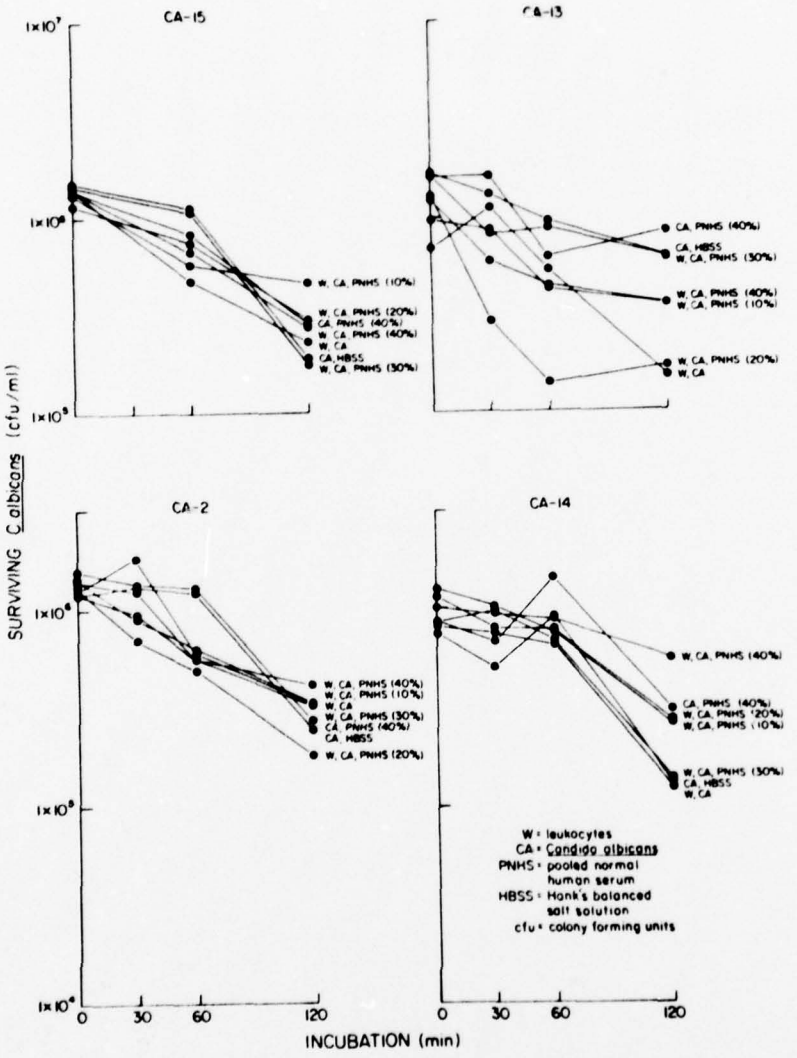


Figure 43. Interaction of pooled normal human serum (PNHS) and PMNs with strains of C. albicans isolated from burned patients and repeatedly subcultured. The following reaction mixtures were tested: 1) PMNs (W), C. albicans (CA); 2) W, CA, PNHS (10%, 20%, 30%, 40%); 3) CA, PNHS (40%); and 4) CA, Hank's balanced salt solution (HBSS). The points represent values of single determinations.

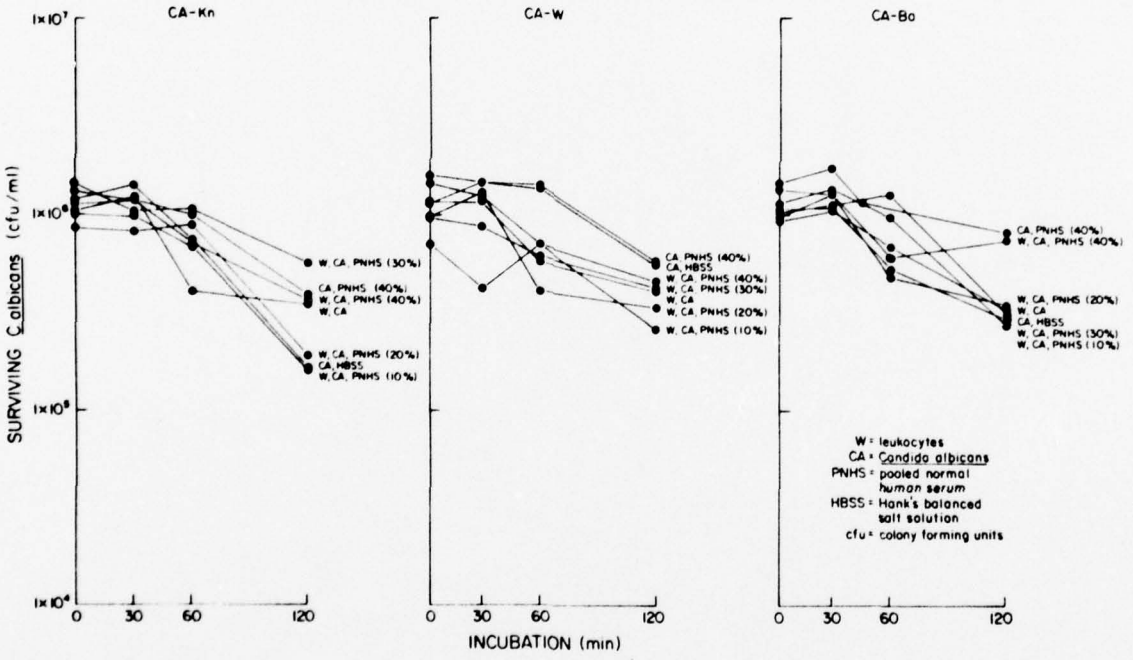


Figure 44. Interaction of pooled normal human serum (PNHS) and PMNs with strains of C. albicans isolated from medical patients. The following reaction mixtures were tested: 1) PMNs (W), C. albicans (CA); 2) W, CA, PNHS (10%, 20%, 30%, 40%); 3) CA, PNHS (40%); and 4) CA, Hank's balanced salt solution (HBSS). The points represent values of single determinations.

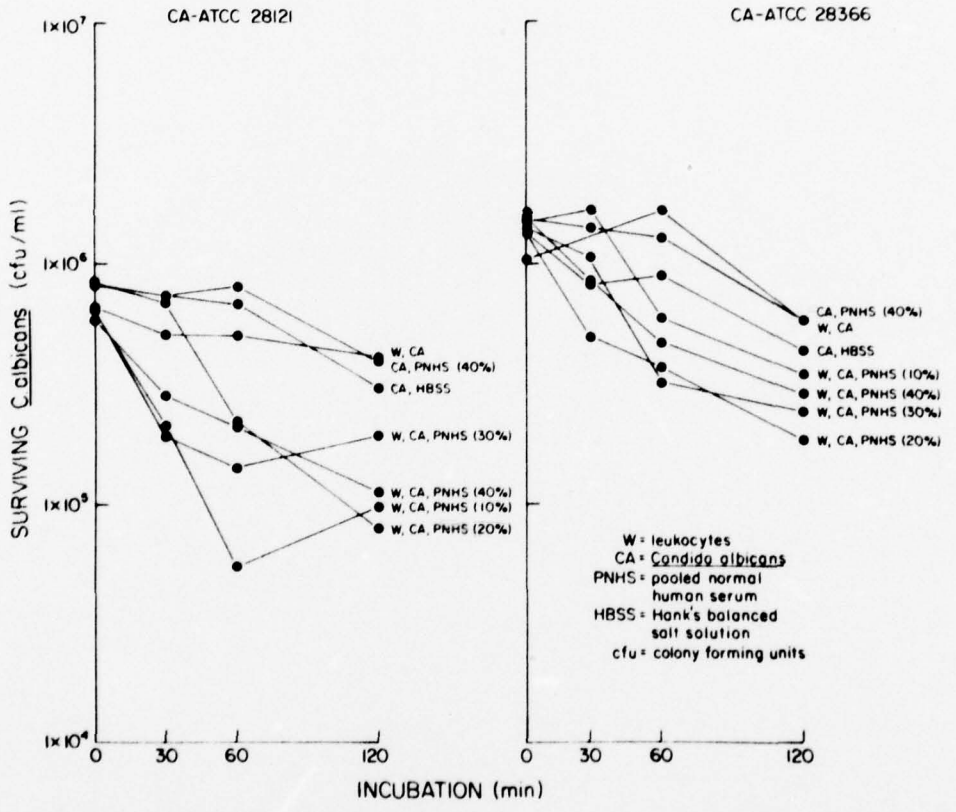


Figure 45. Interaction of pooled normal human serum (PNHS) and PMNs with strains of C. albicans obtained from the American Type Culture Collection. The following reaction mixtures were tested: 1) PMNs (W), C. albicans (CA); 2) W, CA, PNHS (10%, 20%, 30%, 40%); 3) CA, PNHS (40%); and 4) CA, Hank's balanced salt solution (HBSS). The points represent values of single determinations.

candidiasis (106) have suggested that neutrophils are important in host defense against Candida fungemia. Passive transfer of immune serum has been shown to give a significant degree of protection against candidiasis in mice (107). Repeated attempts to transfer resistance by transfer of sensitized lymphoid cells gave negative results, even though cutaneous delayed hypersensitivity was transferred by the cells. The results suggested that cell-mediated immunity was not of primary importance in murine candidiasis, and that humoral immunity contributed to protection.

The most recent in vitro study of phagocytosis of C. albicans yeast forms by PMNs showed that immunoglobulin and the classical and alternative complement pathways in pooled normal human serum were required (108). In this study, phagocytosis was measured with the use of C. albicans radiolabelled with /^{3}H/ adenine, and intracellular killing was not determined.

The interactions of leukocytes with filamentous forms of fungi may not necessarily correlate directly with observations on phagocytosis and killing of yeast forms (101-5,109), especially because hyphae and pseudohyphae are too large to be ingested completely. Diamond et al. have provided evidence for a new leukocyte mechanism whereby human PMNs partially ingested and damaged C. albicans pseudohyphae in the absence of serum (110). Fresh normal human serum or serum heated at 56°C for 30 minutes enhanced damage of the pseudohyphae. In a subsequent publication, these investigators showed that the damage to C. albicans pseudohyphae by PMNs occurred primarily by oxidative mechanisms including the production of superoxide and hydrogen peroxide interacting with myeloperoxidase and halide as well as singlet oxygen (111).

The results of our investigation provide a new dimension to preexisting concepts of resistance to yeast forms of C. albicans. Although our cultures of C. albicans were not examined by phase contrast microscopy, it is doubtful that they contained hyphae or pseudohyphae. Incubation of yeast forms at 37°C for 4 hours in minimal medium without amino acids is required for germ tube formation (110). The strains of C. albicans used in our study were grown in Sabouraud's dextrose broth and were harvested in stationary phase. In addition, the bactericidal assays were carried out in Hank's balanced salt solution utilizing only a 2 hour incubation period at 37°C. ATCC strains of C. albicans isolated from normal human flora were phagocytosed and killed intracellularly by human PMNs in the presence of pooled normal human serum, whereas strains isolated from medical patients or burned patients were more resistant to the bactericidal activity of the PMNs. Moreover, repeated subculture of the clinical isolates did not increase their ability to be killed by the PMNs. Thus, a diversity among strains of C. albicans exists in terms of the ability of PMNs of the host to effectively eliminate the microorganisms.

It is quite possible that the clinical isolates of C. albicans used in our study were effectively phagocytosed, but not killed intracellularly by the PMNs. An alternative hypothesis is that immune antibodies acting alone or synergistically with complement components are required for phagocytosis and intracellular killing of the strains. Our future studies will be directed toward answering these as yet unressolved questions.

5. In vitro interaction of human polymorphonuclear leukocytes, complement, and immunoglobulins with strains of Bacteroides

a. Results

In our previous studies, indirect evidence was provided to indicate that immunoglobulin and alternative complement pathway components were required for opsonization of Bacteroides fragilis and Bacteroides thetaiotaomicron (112). B. fragilis 1365 and B. thetaiotaomicron 1343 were shown to be phagocytosed and killed intracellularly by human polymorphonuclear leukocytes (PMNs) in the presence of pooled normal human serum (PNHS), but not by either PNHS or PMNs alone. Neither strain was phagocytosed and killed intracellularly by PMNs in the presence of hypogammaglobulinemic sera or sera depleted of C3, factors B or D, or terminal complement components C3 to C9. The objectives of the present investigation were as follows: (a) To further assess the requirements for immunoglobulin and components of the alternative complement pathway for phagocytosis and intracellular killing of the Bacteroides strains by PMNs; (b) to determine the role of the polysaccharide capsule in host defense against the Bacteroides strains; (c) to determine if immunoglobulin was required for activation of the alternative complement pathway or for other steps in the opsonic process; and (d) to determine the ability of the lipopolysaccharide of B. fragilis to activate the classical and alternative complement pathways.

Our initial experiments were designed to determine if the opsonic activity of human sera depleted of immunoglobulin or specific alternative complement pathway components, factors B or D, could be restored to normal by supplementing the sera with purified immunoglobulins or factors B and $\overline{D}$ respectively. IgG and IgM were obtained from normal human serum in purified form by the method of Flodin and Killander (113). The fraction containing IgG was further chromatographed on DEAE-cellulose (114). By radial immunodiffusion, the IgG preparation contained 7200 µg/ml of IgG, 120 µg/ml of IgM, and 590 µg/ml of IgA. The IgM preparation contained 2000 µg/ml of IgM; IgG and IgA were undectable in this preparation. Human factor B was purified by the method of Gotze and Muller-Eberhard (87). Human factor $\overline{D}$ was purified by repeated chromatography of normal human serum on Sephadex G-75. The included fractions from the second chromatography were pooled and concentrated. The factor B preparation migrated as a single band on alkaline acrylamide discontinuous electrophoresis at a concentration of 20 µg; the factor $\overline{D}$ preparation migrated as multiple bands at this concentration (115). By radial immunodiffusion, the factor B preparation contained 300 µg/ml of factor B. Utilizing the method of Fearon and Austen, the factor B and factor $\overline{D}$ preparations were found to be active and functionally pure (116). All of the purified immunoglobulin and complement preparations were dialyzed against 0.01 M phosphate buffered saline, pH 7.0, prior to use.

Dose dependent restoration of the opsonic activity of factor B depleted normal human serum {ΔPNHS (50°C, 30 min.)} by purified human factor B is shown in Figure 46. Optimal phagocytosis of both Bacteroides strains by ΔPNHS (50°, 30 min.) was achieved when the serum was supplemented with a physiologic concentration of factor B (300 µg/ml). The opsonic activity of factor D depleted human serum (RD) was not restored to normal by supplementing the serum with a physiologic concentration of purified human factor $\overline{D}$. This finding was not surprising, since purified human factor $\overline{D}$ was also not found to be capable of

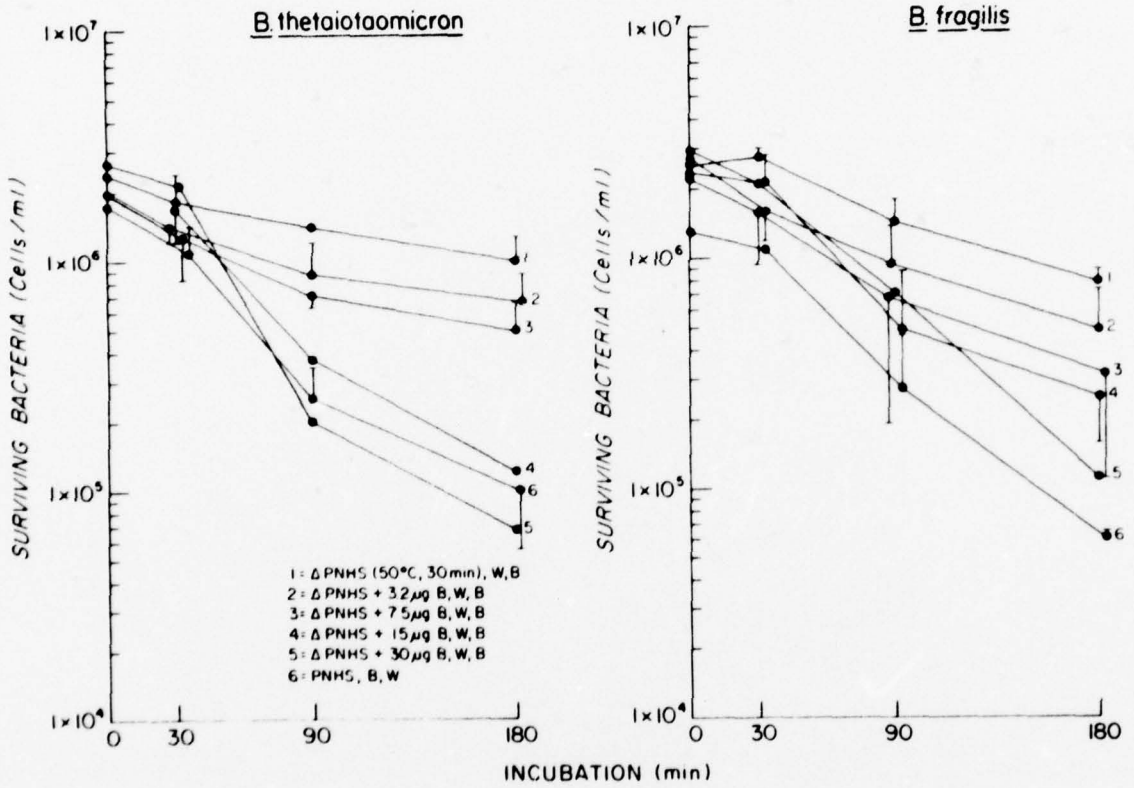


Figure 46. Dose dependent restoration of the opsonic activity of pooled normal human serum heated at 50°C for 30 minutes {ΔPNHS (50°C, 30 min.)} by purified human factor B for the Bacteroides strains. Reaction mixtures consisted of PMNs (W), bacteria (B), and unsupplemented ΔPNHS (50°C, 30 min.) or ΔPNHS (50°C, 30 min.) supplemented with factor B. The control for the experiment was untreated PNHS, W, and B. The points represent mean values of duplicate determinations, and each vertical bar represents the standard error of the mean.

restoring the ability of RD to cause lysis of guinea pig erythrocytes (refer to section 1a). Further testing of our RD preparations has shown that they contain normal classical complement pathway activity and physiologic levels of factor B. Since our factor $\overline{D}$ preparations contain functionally active factor $\overline{D}$, the lack of restoration of C3 conversion in RD by factor $\overline{D}$ reflects a deficiency of other proteins in RD which are essential for alternative pathway activation. The observation that restoration of the opsonic activity of factor B depleted serum was achieved by purified human factor B indicated that factor B participated in opsonization of the Bacteroides strains.

The opsonic activity of hypogammaglobulinemic serum (HS) for the Bacteroides strains was not enhanced by supplementing the serum with physiologic concentrations of purified human IgG (Figure 47). Partial restoration of the opsonic activity of HS for the Bacteroides strains was achieved by supplementing the serum with purified human IgM (Figure 48). These results indicated that neither IgG nor IgM acted alone to promote phagocytosis of the Bacteroides strains in the presence of complement. The opsonic activity of IgA deficient human serum (IgAdHS) for the Bacteroides strains was next investigated. The opsonic activity of IgAdHS and PNHS for both Bacteroides strains was found to be equivalent (Figure 49). These results indicated that IgA was not required for phagocytosis and intracellular killing of the strains by PMNs. The finding that partial restoration of the opsonic activity of HS was achieved by supplementing the serum with purified human IgM suggested that synergism between IgG and IgM might be required for optimal phagocytosis and intracellular killing of the Bacteroides strains by PMNs.

Our next experiments were designed to determine the role of the polysaccharide capsule in host defense against the Bacteroides strains. Kasper has proposed that capsular polysaccharide contributes to the virulence of B. fragilis by acting as an antiphagocytic agent (61). Our observations that B. fragilis 1365 and B. thetaiotaomicron 1343 had similar opsonic requirements led us to consider the possibility that both isolates were encapsulated. This concept or the concept that neither strain was encapsulated might explain why the interaction of the strains with serum factors and PMNs was identical. Since polysaccharide capsules cannot be visualized with routine staining techniques, ruthenium red staining was utilized. Washed cells of B. fragilis 1365 and B. thetaiotaomicron 1343 were prepared, fixed, stained, and visualized as described by Kasper (61). A representative electron micrograph of B. fragilis 1365 stained with ruthenium red is shown in Figure 50. Capsular polysaccharide external to the outer membrane was not observed. However, a thick capsule was observed on B. thetaiotaomicron 1343 (Figure 51). The observation that B. thetaiotaomicron 1343 was encapsulated and B. fragilis 1365 was not, provided preliminary evidence to indicate that capsular polysaccharide was not anti-phagocytic.

It was next of interest to determine if B. fragilis 1365 and B. thetaiotaomicron 1343 used in our studies were representative clinical isolates of B. fragilis and B. thetaiotaomicron in terms of their in vitro interaction with PNHS and human PMNs. Clinical isolates of B. fragilis and B. thetaiotaomicron were obtained from the Surgical Bacteriology Laboratory, Surgical Research Unit, Cincinnati, O. and from the American Type Culture Collection, Rockville, Md. and designated SRU and ATCC respectively. The ability of PNHS and PMNs to promote killing of the Bacteroides strains either alone or in combination was determined.

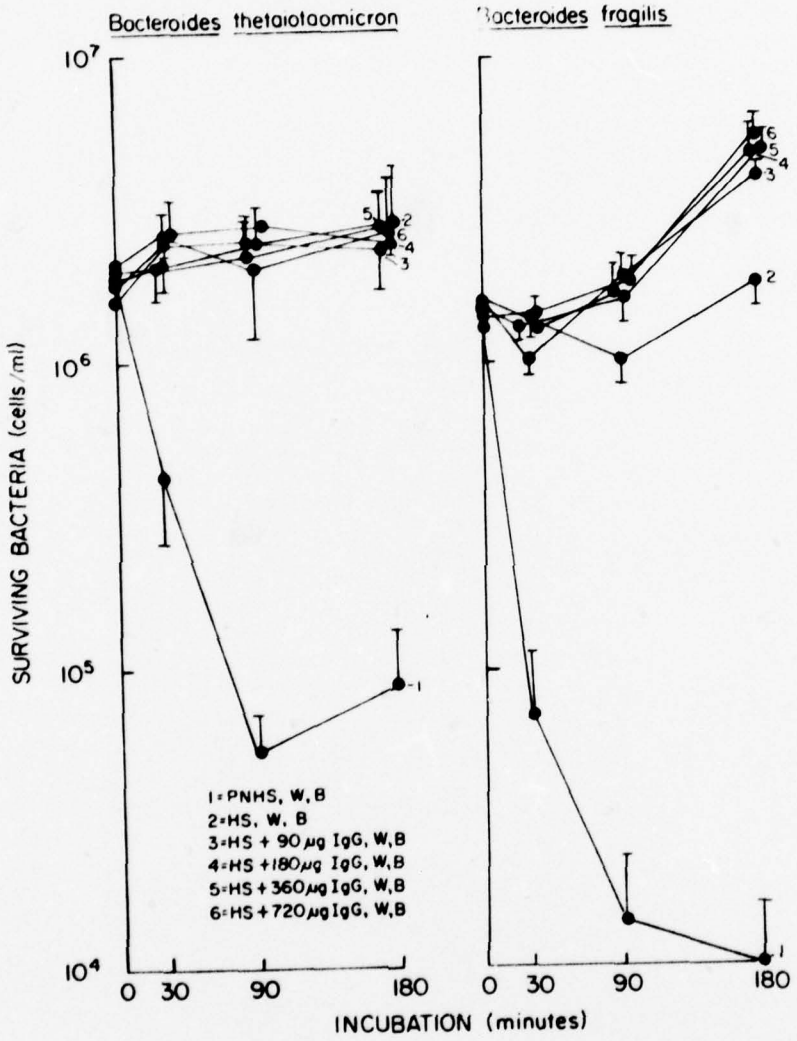


Figure 47. Opsonic activity of hypogammaglobulinemic serum (HS) supplemented with increasing concentrations of purified human IgG for the Bacteroides strains. Reaction mixtures consisted of PMNs (W), bacteria (B), and unsupplemented HS or HS supplemented with IgG. Control for the experiment was pooled normal human serum (PNHS), W, and B. The points represent mean values of duplicate determinations, and each vertical bar represents the standard error of the mean.

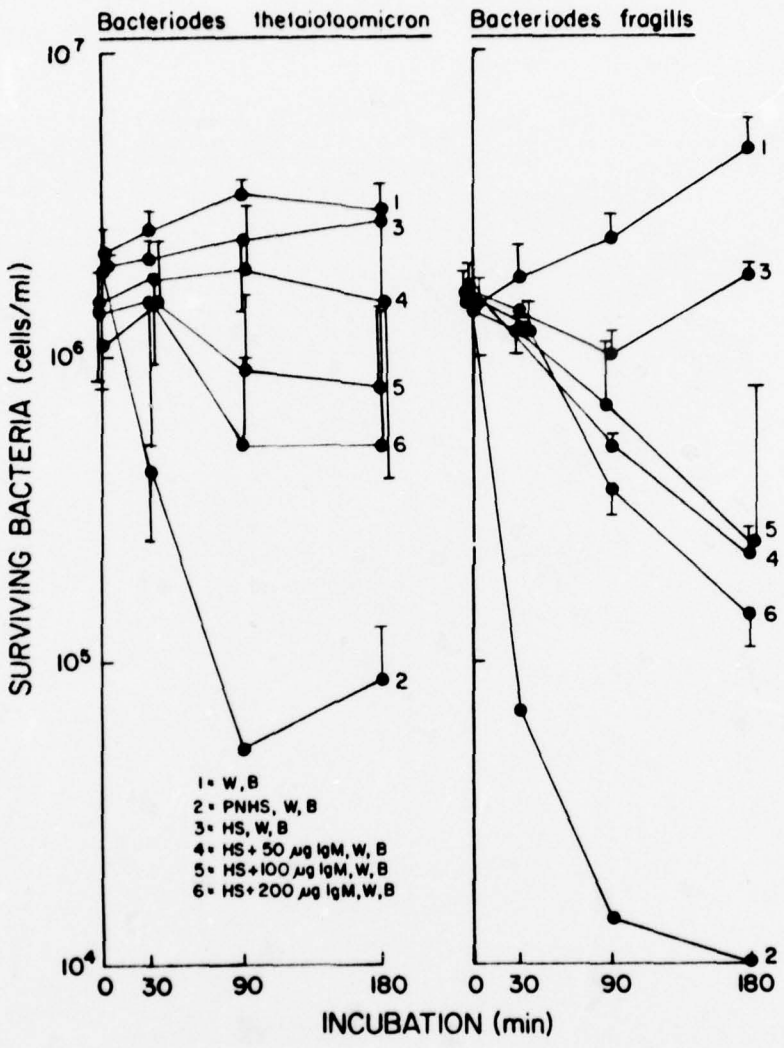


Figure 48. Opsonic activity of hypogammaglobulinemic serum (HS) supplemented with increasing concentrations of purified human IgM for the Bacteroides strains. Reaction mixtures consisted of PMNs (W), bacteria (B), and unsupplemented HS or HS supplemented with IgM. Controls for the experiment were as follows: 1) W and B; and 2) pooled normal human serum (PNHS), W, and B. The points represent mean values of duplicate determinations, and each vertical bar represents the standard error of the mean.

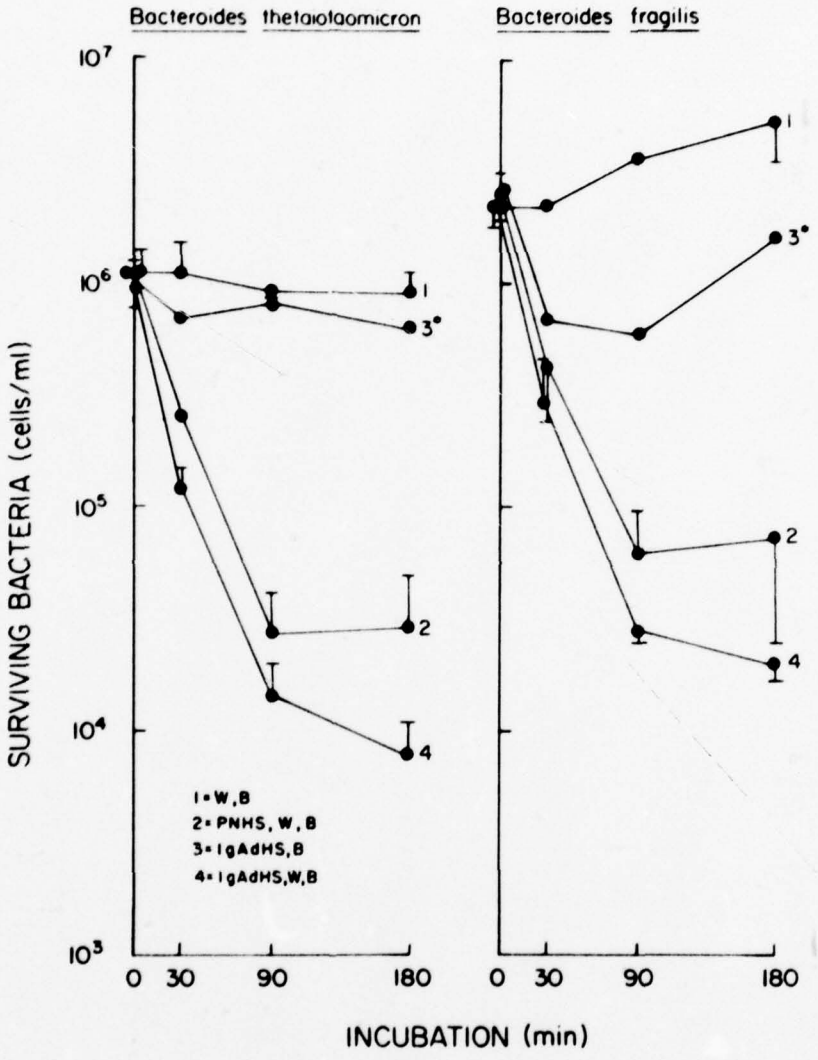


Figure 49. Opsonic activity of IgA deficient human serum (IgAdHS) for the Bacteroides strains. Reaction mixtures consisted of IgAdHS, PMNs (W), and bacteria (B), or IgAdHS and B alone in the absence of PMNs. Controls for the experiment were as follows: 1) W and B; and 2) pooled normal human serum (PNHS), W, and B. The points represent mean values of duplicate determinations, and each vertical bar represents the standard error of the mean. The asterisks indicate that the values were obtained from single determinations.

Figure 50. Electron micrograph of B. fragilis 1365 stained with ruthenium red for visualization of polysaccharide capsule (X 130,000). (Refer to page 114a for the figure.)

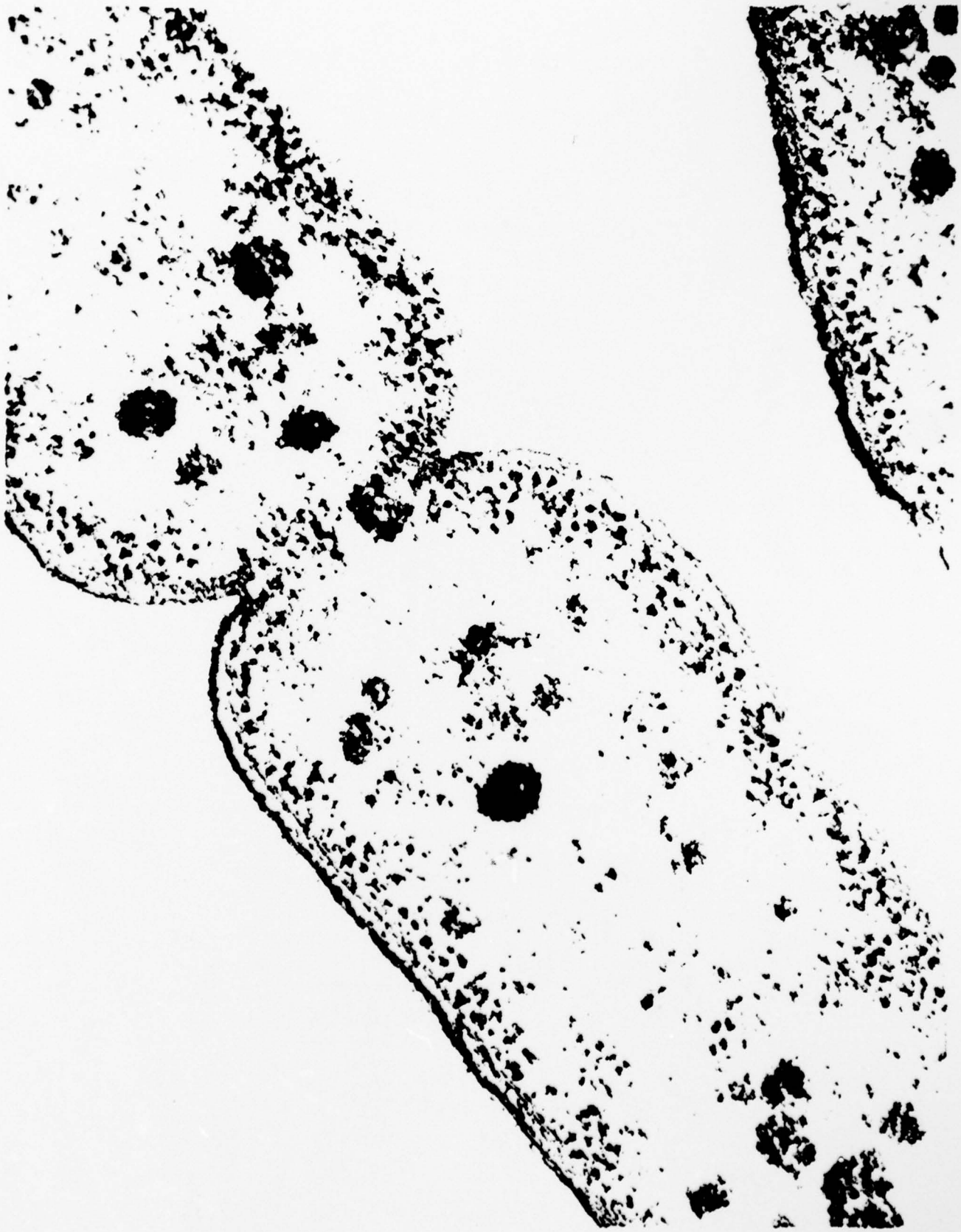
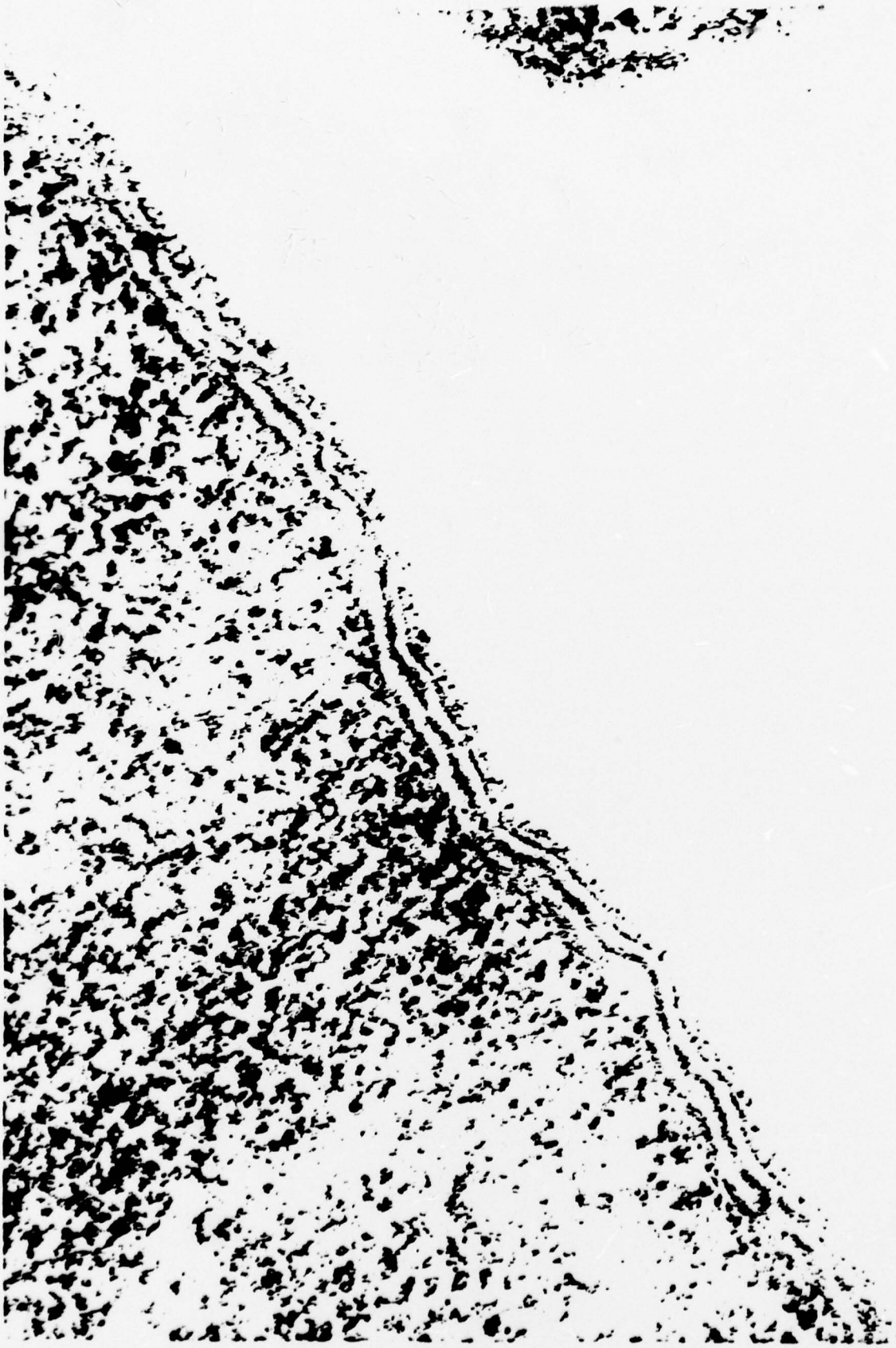


Figure 51. Electron micrograph of *B. thetaiotaomicron* 1343 stained with ruthenium red for visualization of polysaccharide capsule (X 130,000). (Refer to page 115a for the figure.)



Experimental conditions, serum concentration, and the PMN/bacteria ratio were identical to those used in the B. fragilis 1365 and B. thetaiotaomicron 1343 bactericidal assays. All of the B. fragilis strains were phagocytosed and killed intracellularly in the presence of PNHS and PMNs, but not by PMNs alone (Figure 52). Three of the four strains were also killed to some extent in the presence of PNHS alone. All of the clinical isolates of B. thetaiotaomicron as well as a fecal isolate of B. thetaiotaomicron (ATCC 8492) were killed by PMNs and PNHS, but not by either PMNs or PNHS alone (Figure 53). These results indicated that clinical isolates of B. fragilis and B. thetaiotaomicron were phagocytosed and killed intracellularly by PMNs only in the presence of PNHS; several B. fragilis strains were also sensitive to killing by serum in the absence of PMNs.

Previous studies have indicated that fecal isolates of B. fragilis and B. thetaiotaomicron were sensitive to killing by serum in the absence of PMNs, whereas clinical isolates were not (117). Since one fecal isolate of B. thetaiotaomicron used in our study was not found to be susceptible to direct killing by PNHS, it was of interest to determine the in vitro interaction of PMNs and PNHS with other fecal isolates of Bacteroides. B. distasonis ATCC 8503 and B. vulgatus ATCC 8482 were not found to be killed by PNHS alone (Figure 54). Both strains were killed intracellularly by PMNs only in the presence of PNHS. These results refuted the concept that fecal and clinical isolates of Bacteroides differed in terms of their susceptibility to killing by serum in the absence of PMNs. Since previous studies have shown that neither B. distasonis ATCC 8503 nor B. vulgatus ATCC 8482 is encapsulated (61), our results also provided further support for the concept that capsular polysaccharide is not anti-phagocytic.

The next objective of our studies was to determine if immunoglobulin was required for activation of the alternative complement pathway or for other steps in the opsonic process. Washed bacterial cells of B. fragilis 1365 and B. thetaiotaomicron 1343 were tested for their ability to initiate C3 conversion and C3 to C9 consumption in untreated PNHS or HS and in PNHS or HS treated with 10 mM ethylene glycol tetra-acetic acid (EGTA) and 10 mM $MgCl_2$. The EGTA-treated magnesium supplemented sera were referred to as MgEGTA-PNHS and MgEGTA-HS respectively. Treatment of the sera with MgEGTA was used to block classical pathway activity as previously described in sections A1, A2, C1, and C2 of this report. Washed cells in saline at final concentrations of 1.0×10^9 or 1.0×10^{10} cells/ml and sera were incubated for 1 hour at 37°C. The cells were then deposited by centrifugation, and residual B antigen of C3 and hemolytic C3 to C9 were measured and compared to the concentrations of B antigen of C3 and C3 to C9 in saline treated sera. Inulin, a polyfructose which activates the alternative pathway in the absence of immunoglobulin, was included for comparative purposes. As shown in Table 18, C3 conversion by inulin was equivalent in PNHS, MgEGTA-PNHS, HS, and MgEGTA-HS. In contrast, B. fragilis 1365 and B. thetaiotaomicron 1343 converted C3 less efficiently in MgEGTA-PNHS, HS, and MgEGTA-HS than in untreated PNHS. Conversion of C3 by B. thetaiotaomicron 1343 in HS and MgEGTA-HS was equivalent; C3 conversion was only slightly reduced in MgEGTA-HS when B. fragilis 1365 was used as the activating substance. These results indicated that B. fragilis 1365 and B. thetaiotaomicron 1343 activated the classical as well as the alternative complement pathway, and that alternative pathway activation by the bacterial strains was dependent on minimal or non-existent amounts of immunoglobulin. Inulin also converted C3 via the alternative

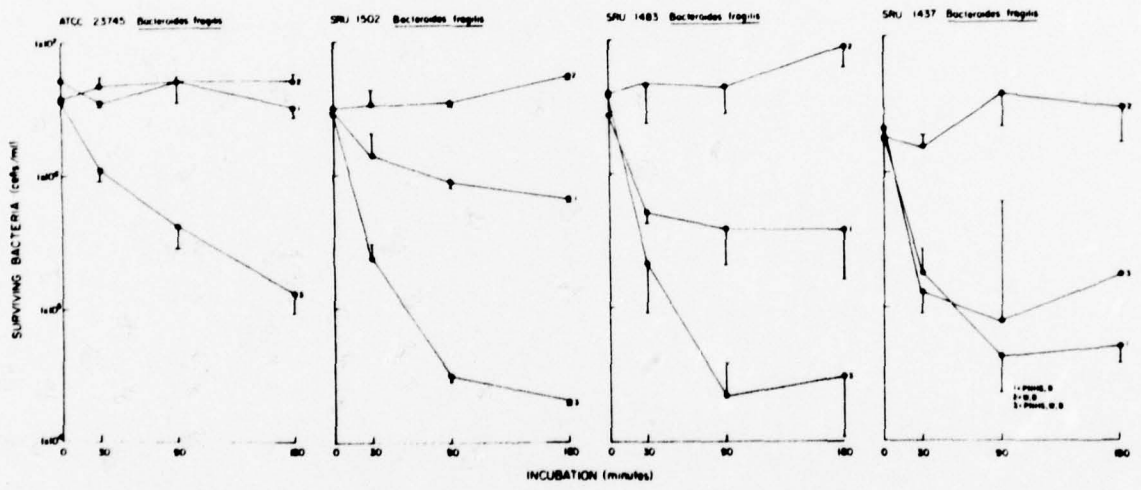


Figure 52. In vitro interaction of human polymorphonuclear leukocytes (PMNs) and pooled normal human serum (PNHS) with clinical isolates of B. fragilis obtained from the Surgical Bacteriology Laboratory (SRU) and the American Type Culture Collection (ATCC). Reaction mixtures were as follows: 1) PNHS and bacteria (B); 2) PMNs (W) and B; and 3) PNHS, W, and B. The points represent mean values of duplicate determinations, and each vertical bar represents the standard error of the mean.

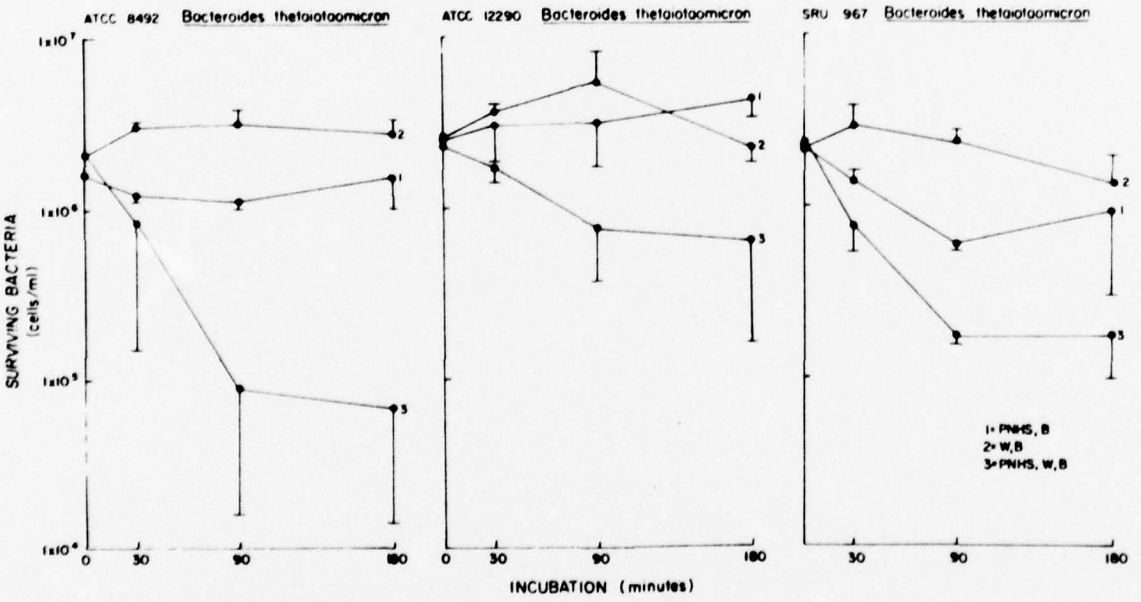


Figure 53. In vitro interaction of human polymorphonuclear leukocytes (PMNs) and pooled normal human serum (PNHS) with isolates of B. thetaiotaomicron obtained from the Surgical Bacteriology Laboratory (SRU) and the American Type Culture Collection (ATCC). All of the strains were clinical isolates, with the exception of ATCC 8492 which was isolated from human feces. Reaction mixtures were as follows: 1) PNHS and bacteria (B); 2) PMNs (W) and B; and 3) PNHS, W, and B. The points represent mean values of duplicate determinations, and each vertical bar represents the standard error of the mean.

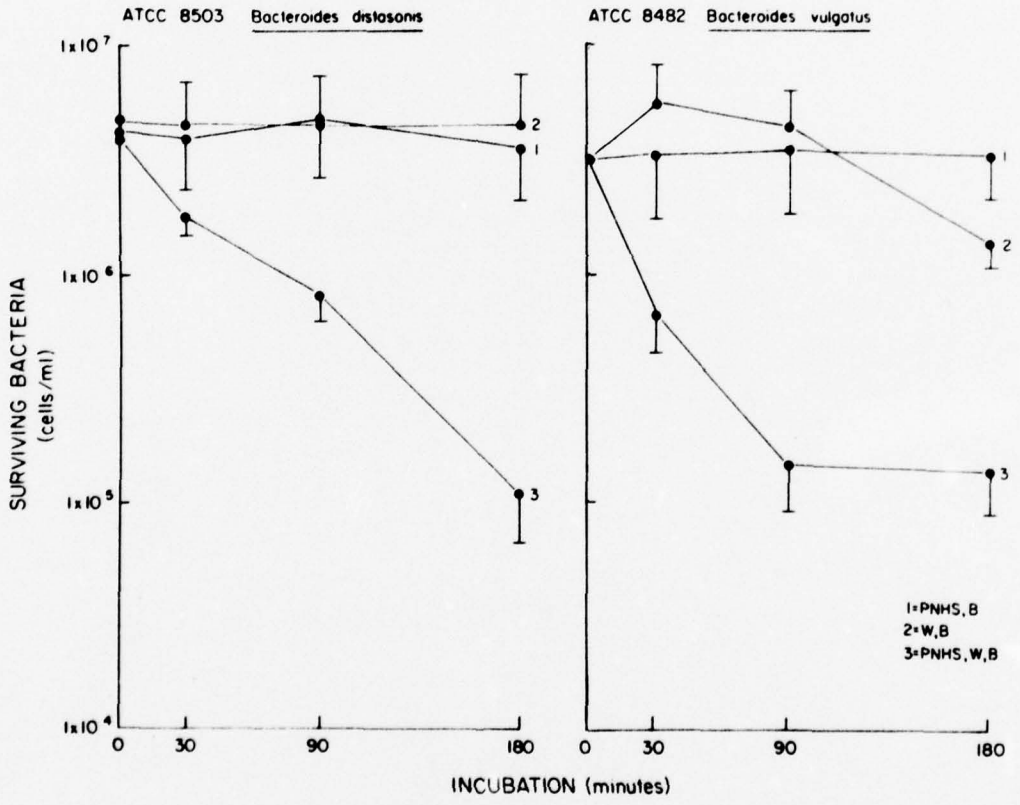


Figure 54. In vitro interaction of human polymorphonuclear leukocytes (PMNs) and pooled normal human serum (PNHS) with fecal isolates of B. distasonis and B. vulgatus obtained from the American Type Culture Collection (ATCC). Reaction mixtures were as follows: 1) PNHS and bacteria (B); 2) PMNs (W) and B; and 3) PNHS, W, and B. The points represent mean values of duplicate determinations, and each vertical bar represents the standard error of the mean.

Table 18. Conversion of C3 by Heat-Killed Washed Cells or Untreated Washed Cells of *B. fragilis* and *B. thetaiotaomicron* in Human Sera Depleted of the Classical Complement Pathway and/or Immunoglobulin

Activating Substance	C3 Conversion (%)[a]							
	PNHS[b]		MgEGTA-PNHS[c]		HS[d]		MgEGTA-HS[e]	
	10^9[f]	10^{10}[f]	10^9[f]	10^{10}[f]	10^9[f]	10^{10}[f]	10^9[f]	10^{10}[f]
B. fragilis 1365	66	78	30	50	50	65	34	58
B. fragilis 1365[g]	30	40	-	-	-	-	-	-
B. thetaiotaomicron 1343	58	-[i]	46	-	55	-	68	-
B. thetaiotaomicron 1343[g]	36	-	-	-	-	-	-	-
Inulin[h]	58	-	54	-	55	-	66	-

[a]Values of single determinations are presented.

[b]PNHS = pooled normal human serum.

[c]MgEGTA-PNHS = pooled normal human serum treated with 10 mM EGTA and 10 mM $MgCl_2$.

[d]HS = hypogammaglobulinemic serum.

[e]MgEGTA-HS = hypogammaglobulinemic serum treated with 10 mM EGTA and 10 mM $MgCl_2$.

[f]Final concentration of microorganisms (cells/ml) tested in the assay system.

[g]Washed bacterial cells were heat-killed at 100°C for 1 hour.

[h]Final concentration of inulin was 10 mg/ml.

[i]Minus sign indicates that the assay was not performed.

pathway in the presence of minimal immunoglobulin; however this activating substance appeared to activate only the alternative pathway. When washed cells of B. fragilis and B. thetaiotaomicron were heat-killed at 100°C for 1 hour, their ability to initiate C3 conversion was markedly reduced (Table 18). These results provided indirect evidence to indicate that outer membrane protein on the surface of Bacteroides, destroyed by heat denaturation, might be required for optimal complement activation.

Identical results were obtained when washed bacterial cells and inulin were tested for their ability to initiate C3 to C9 consumption in sera depleted of immunoglobulin and/or classical pathway activity. C3 to C9 consumption by the bacteria was less efficient in MgEGTA-PNHS, HS, and MgEGTA-HS than in untreated PNHS, although C3 to C9 consumption by the bacteria in HS and MgEGTA-HS was equivalent (Table 19). In contrast, C3 to C9 consumption by inulin was equivalent in PNHS, MgEGTA-PNHS, HS, and MgEGTA-HS. These results provided further support for the concept that B. fragilis 1365 and B. thetaiotaomicron 1343 activated the classical as well as the alternative pathway, whereas inulin activated only the alternative pathway. Moreover, alternative pathway activation by the bacteria and inulin required minimal immunoglobulin, if any. Thus, immunoglobulin was required for opsonization of B. fragilis 1365 and B. thetaiotaomicron 1343 primarily for one or more steps in the opsonic process other than alternative complement pathway activation.

The ability of lipopolysaccharide (LPS) prepared from B. fragilis 1365 to activate the alternative complement pathway was next investigated. LPS was prepared by phenol-water extraction and tested prior to and after RNAse treatment as described in section 3c of this report. LPS prior to RNAse treatment (LPS-1) initiated C3 to C9 consumption in PNHS more efficiently than LPS after RNAse treatment (LPS-2) (Table 20). C3 to C9 consumption in MgEGTA-PNHS by LPS-1 and LPS-2 was markedly reduced. These results indicated that LPS prepared from B. fragilis 1365 activated the classical as well as the alternative complement pathway.

b. Discussion

From the data presented in this investigation, several preliminary conclusions can be drawn as follows: (a) Immunoglobulin as well as components of the alternative complement pathway were required for phagocytosis and intracellular killing of B. fragilis 1365 and B. thetaiotaomicron 1343 by human PMNs; (b) neither IgG nor IgM acted alone to promote phagocytosis and intracellular killing of the Bacteroides strains by PMNs in the presence of complement; (c) IgA did not participate in phagocytosis and intracellular killing of the Bacteroides strains by PMNs; (d) immunoglobulin was not shown to be required for alternative pathway activation by the Bacteroides strains, and therefore must be required for other steps in the opsonic process; (e) encapsulated and nonencapsulated strains of Bacteroides interacted identically with PNHS and PMNs in vitro, suggesting that capsular polysaccharide was not a determining factor in host defense against these microorganisms; and (f) although the lipopolysaccharide of B. fragilis was capable of activating the classical as well as the alternative complement pathway, protein or some other heat-labile cell wall component on the surface of Bacteroides appeared to be equally if not more important in initiating complement activation.

Table 19. Consumption of C3 to C9 by Untreated Washed Cells of B. fragilis and B. thetaiotaomicron in Human Sera Depleted of the Classical Complement Pathway and/or Immunoglobulin

Activating Substance	C3 to C9 Consumption (%)[a]							
	PNHS[b]		MgEGTA-PNHS[c]		HS[d]		MgEGTA-HS[e]	
	10^{9f}	10^{10f}	10^{9f}	10^{10f}	10^{9f}	10^{10f}	10^{9f}	10^{10f}
B. fragilis 1365	86	86	63	81	55	72	65	77
B. thetaiotaomicron 1343	89	-[h]	61	-	59	-	63	-
Inulin[g]	44	-	72	-	41	-	83	-

[a]Values of single determinations are presented.

[b]PNHS = pooled normal human serum.

[c]MgEGTA-PNHS = pooled normal human serum treated with 10 mM EGTA and 10 mM $MgCl_2$.

[d]HS = hypogammaglobulinemic serum.

[e]MgEGTA-HS = hypogammaglobulinemic serum treated with 10 mM EGTA and 10 mM $MgCl_2$.

[f]Final concentration of microorganisms (cells/ml) tested in the assay system.

[g]Final concentration of inulin was 10 mg/ml.

[h]Minus sign indicates that the assay was not performed.

Table 20. C3 to C9 Consumption by Lipopolysaccharide (LPS) Prepared from B. fragilis 1365 in PNHS[a] and MgEGTA-PNHS[b]

	C3 to C9 Consumption (%)[c]			
	PNHS		MgEGTA-PNHS	
	1 mg/ml[d]	5 mg/ml[d]	1 mg/ml[d]	5 mg/ml[d]
LPS-1[e]	55	71	4	45
LPS-2[f]	41	69	29	40

[a]PNHS = pooled normal human serum.

[b]MgEGTA-PNHS = pooled normal human serum treated with 10 mM EGTA and 10 mM $MgCl_2$.

[c]Values of single determinations are presented.

[d]Final concentrations of LPS-1 and LPS-2 tested in the assay system.

[e]LPS-1 was phenol-water extracted ethanol precipitated LPS prior to RNAse treatment.

[f]LPS-2 was phenol-water extracted ethanol precipitated LPS after RNAse treatment.

The observation that neither IgG nor IgM at physiologic concentrations restored the opsonic activity of immunoglobulin depleted serum for B. fragilis and B. thetaiotaomicron suggested that these immunoglobulins may act synergistically to promote opsonization. In addition, the observation that the alternative complement pathway was utilized during the opsonic process does not exclude participation of the classical complement pathway. Our studies have shown that washed cells of B. fragilis and B. thetaiotaomicron were capable of activating both complement pathways when added directly to serum, and therefore the classical pathway may also be utilized during opsonization of these microorganisms.

Our conclusion that the polysaccharide capsule did not participate in host defense against Bacteroides was based on the observations that encapsulated B. thetaiotaomicron 1343 and non-encapsulated B. fragilis 1365 had similar opsonic requirements and that isolates of B. fragilis, B. thetaiotaomicron, B. vulgatus, and B. distasonis did not differ in terms of their in vitro interaction with human PMNs and PNHS. Kasper has shown that antisera prepared against B. thetaiotaomicron ATCC 8492 and ATCC 12290, B. vulgatus ATCC 8482, and B. distasonis ATCC 8503 did not contain antibodies to capsular polysaccharide, whereas antisera to B. fragilis ATCC 23745 contained antibodies which were directed against capsular polysaccharide (61). Moreover, capsular polysaccharide was demonstrated on the surface of B. fragilis ATCC 23745 by electron microscopy using ruthenium red staining. In contrast, Babb and Cummins detected capsules by using india ink staining and phase contrast microscopy on isolates of B. fragilis, B. thetaiotaomicron, B. vulgatus, and B. ovatus, but not on B. distasonis (118). These investigators also showed that there was no correlation between the presence of capsular material and virulence. The ATCC isolates of B. fragilis, B. thetaiotaomicron, B. distasonis, and B. vulgatus used by Kasper were found in our study to be phagocytosed and killed intracellularly by PMNs only in the presence of PNHS, and not by either PMNs or PNHS alone. If it is verified that only B. fragilis ATCC 23745 is encapsulated, then our results provide further support to the concept that capsular polysaccharide of Bacteroides is not anti-phagocytic and may contribute to virulence by another mechanism.

In our investigation, three clinical isolates of B. fragilis were the only strains of Bacteroides to be killed to some extent by PNHS in the absence of PMNs. All of the other strains of Bacteroides used in our study including fecal isolates were not sensitive to killing by serum. Our results are therefore not in agreement with those of Casciato et al. who showed that fecal isolates of B. fragilis and B. thetaiotaomicron were sensitive to killing by serum, whereas clinical isolates of B. fragilis and B. thetaiotaomicron were not (117).

Our results showing that immunoglobulin was not required for alternative pathway activation by B. fragilis and B. thetaiotaomicron are identical to those obtained with gram-negative aerobic bacilli (refer to section C2). However, the results on the Bacteroides strains require verification, since only single determinations were performed. It will also be of considerable interest to determine the role of immunoglobulin in the opsonic process, since immunoglobulin does not appear to be required in large amounts for complement activation.

Further studies on the moiety of Bacteroides responsible for complement activation are also necessary. Dr. Dennis Kasper had promised to send us capsular polysaccharide, outer membrane protein, lipopolysaccharide, and lipid A

prepared from B. fragilis ATCC 23745; however, his own research efforts with the reagents did not permit him to do so. Therefore, we have initiated purification of the cell wall components, and hope to extend the preliminary observations described in this investigation.

prepared from B. fragilis ATCC 23745; however, his own research efforts with the reagents did not permit him to do so. Therefore, we have initiated purification of the cell wall components, and hope to extend the preliminary observations described in this investigation.

V. CONCLUSIONS

A. Reduction in C3 conversion in patients with severe thermal injury was shown to be caused by a deficiency of proteins required for alternative complement pathway activation, rather than to an inhibitor of C3 conversion. No correlation was demonstrated between this humoral abnormality and the occurrence, duration, or outcome of septicemia.

B. Consumption of the classical complement pathway occurred preferentially prior to and during septicemia in thermally injured patients. Reduction in classical pathway activity was not demonstrated in non-septic burned patients, suggesting that this humoral abnormality was predictive of septic episodes. Alternative pathway consumption occurred infrequently during septicemia and appeared to result from generation of C3b via consumption of the classical pathway. Consumption of components of the alternative and/or classical complement pathway occurring during septicemia did not decrease the opsonic capacity of the patients' sera for their own infecting microorganisms.

C. Multiple abnormalities of the classical and alternative complement pathways, immunoglobulins, and opsonins were shown to occur following abdominal trauma, some of which persisted after the first week post trauma. The humoral abnormalities in the abdominal trauma patients were not found to be related to surgical procedures or to fluid imbalances. Patients who subsequently developed microbial infections were the only patients who had decreased classical pathway activity that appeared to result from consumption of components. Reduction in IgM occurring during the initial post trauma period was not found to be related to splenectomy.

D. A heterogeneity in the requirements for immunoglobulin and the alternative and classical complement pathways for phagocytosis and intracellular killing of clinical isolates of E. coli, P. mirabilis, K. pneumoniae, and S. marcescens by human PMNs was demonstrated. Strains isolated from burned patients did not demonstrate a unique pattern of opsonic requirements, in comparison to the same species isolated from other sources. The primary role of immunoglobulin in the opsonic process was shown to be for steps other than complement activation.

E. Purified lipid A and LPS were shown to activate terminal complement components in normal human or guinea pig sera equally efficiently. Other heat-stable components on the bacterial cell surface also appeared to be involved in complement activation. LPS was shown to activate the alternative and classical complement pathways, whereas lipid A activated only the classical pathway.

F. Immunoglobulin as well as components of the alternative complement pathway were shown to be required for phagocytosis and intracellular killing of B. fragilis and B. thetaiotaomicron by human PMNs. Neither IgG nor IgM acted alone to promote phagocytosis and intracellular killing of B. fragilis and B. thetaiotaomicron by human PMNs in the presence of complement. IgA did not participate in phagocytosis and intracellular killing of the Bacteroides strains. Immunoglobulin was not shown to be required for alternative pathway activation by the Bacteroides strains, and therefore must be required for other steps in the opsonic process. Encapsulated and non-encapsulated strains of Bacteroides interacted identically with PNHS and

PMNs *in vitro*, suggesting that capsular polysaccharide was not a determining factor in host defense against these microorganisms. Although the lipopolysaccharide of *B. fragilis* was shown to be capable of activating the classical as well as the alternative complement pathway, protein or some other heat-labile component on the surface of *Bacteroides* appeared to be equally if not more important in initiating complement activation.

G. Strains of *C. albicans* isolated from normal human flora were shown to be phagocytosed and killed intracellularly by human PMNs in the presence of pooled normal human serum, whereas strains isolated from burned patients or medical patients were resistant to the bactericidal activity of the PMNs.

VI. LITERATURE CITED

1. Altemeier, W.A., Hummel, R.P., Hill, E.O., and Lewis, S., Ann. Surg. 178:436, 1973.

2. Altemeier, W.A., Culbertson, W.R., and Hummel, R.P., Surg. Clin. No. Am. 48:227, 1968.

3. Salaki, J.S., Black, R., Tally, F.P., and Kislack, J.W., Am. J. Med. 60:426, 1976.

4. Alexander, J.W., and Meakins, J.L., Ann. Surg. 176:273, 1972.

5. Rittenbury, M.S., and Hanback, L.D., J. Trauma 7:523, 1967.

6. Munster, A.M., Eurenius, K., Katz, M., Canales, L., Foley, F.D., and Mortensen, R.F., Ann. Surg. 177:139, 1973.

7. Bjornson, A.B., and Alexander, J.W., J. Lab. Clin. Med. 83:372, 1974.

8. Bjornson, A.B., Altemeier, W.A., and Bjornson, H.S., J. Trauma 16:905, 1976.

9. Bjornson, A.B., Altemeier, W.A., and Bjornson, H.S., Ann. Surg. 186:88, 1977.

10. Arturson, G., Hogman, C.F., Johansson, S.G.O., and Killander, J., Lancet 1:546, 1969.

11. Furstenberg, H.S., Bruns Beitr. Klin. Chir. 212:481, 1966.

12. Kohn, J., and Cort, D.F., Lancet 1:836, 1969.

13. Munster, A.M., and Hoagland, H.C., Surg. Forum 20:76, 1969.

14. Ritzmann, S., McClung, C., Falls, D., Larson, D.L., Abston, S., and Goldman, A.S., Lancet 1:1152, 1969.

15. Fjellstrom, K.E., and Arturson, G., Acta. Path. Microbiol. Scand. 59:257, 1963.

16. Bjornson, A.B., Altemeier, W.A., Bjornson, H.S., Iserson, M.L., and Tang, T., Ann. Surg., in press.

17. Jasin, H.E., J. Immunol. 109:26, 1972.

18. Messner, R.P., and Jelinek, J., J. Clin. Invest. 49:2165, 1970.

19. Medicus, R.G., Schreiber, R.D., Gotze, O., and Muller-Eberhard, H.J., P.N.A.S. 73:612, 1976.

20. Schreiber, R.D., Gotze, O., and Muller-Eberhard, H.J., J. Exp. Med. 144:1062, 1976.

21. Medicus, R.G., Gotze, O., and Muller-Eberhard, H.J., J. Exp. Med. 144:1076, 1976.

22. Whaley, K., and Ruddy, S., J. Exp. Med. 144:1147, 1976.

23. Weiler, J.M., Daha, M.R., Austen, K.F., and Fearon, D.T., P.N.A.S. 73:3268, 1976.

24. Pangburn, M.K., Schreiber, R.D., and Muller-Eberhard, H.J., J. Exp. Med. 146:257, 1977.

25. Bjornson, A.B., Altemeier, W.A., and Bjornson, H.S., Ann. Surg., in press.

26. Hunsicker, L.G., Ruddy, S., and Austen, K.F., J. Immunol. 110:128, 1973.

27. Wasi, C., Wasi, P., and Thongcharoen, P., Lancet 2:237, 1971.

28. Carlisle, H.N., and Saslaw, S., Proc. Soc. Exp. Biol. Med. 102:150, 1959.

29. Gavrilis, P., Rothenberg, S.P., and Guy, R., Am. J. Med. 57:542, 1974.

30. Schumacher, M.J., Arch. Dis. Child. 45:114, 1970.

31. Conolly, W.B., Hunt, T.K., Sonne, M., and Dunphy, J.E., Surg. Gynecol. Obstet. 128:713, 1969.

32. Cuthbertson, D., Filstone, W.J., and Green, J.A., Lancet 1:987, 1969.

33. Havens, W.P., Jr., Bach, D.G., and Siegel, I., J. Clin. Invest. 33:940, 1954.

34. Balch, H.H., Ann. Surg. 157: 1, 1963.

35. Blattberg, B., and Levy, M.N., Am. J. Physiol. 203:867, 1962.

36. Blattberg, B., and Levy, M.N., Am. J. Physiol. 209:71, 1965.

37. Blattberg, B., Frosolono, M.F., Misra, R.P. and Levy, M.N., J. Retic. Soc. 12:371, 1972.

38. Schildt, B.E., Acta. Chir. Scand. 136:359, 1970.

39. Altura, B.M. and Hershey, S.G., Am. J. Physiol. 215:1414, 1968.

40. Altura, B.M., Thaw, C., Dyce, D., and Hershey, S.G., Fed. Proc. 26:332, 1967.

41. Balch, H.H., Ann. Surg. 142:145, 1955.

42. Alexander, J.W., Hegg, M., and Altemeier, W.A., Ann. Surg. 168:447, 1968.

43. Munster, A.M., Eurenius, K., Mortensen, R., and Mason, A.D., Jr., Transplantation 14:106, 1972.

44. McCabe, W.R., New Engl. J. Med. 288:21, 1973.

45. Fearon, D.T., Ruddy, S., Schur, P., and McCabe, W.R., New Engl. J. Med. 292:937, 1975.

46. Gewurz, H., Mergenhagen, S.E., Nowotny, A., and Phillips, J.K., J. Bacteriol. 95:397, 1968.

47. Gewurz, H., Shin, H.S., and Mergenhagen, S.E., J. Exp. Med. 128:1049, 1968.

48. Frank, M.M., May, J.F., Gaither, T., and Ellman, L., J. Exp. Med. 134:176, 1971.

49. Galanos, C., Rietschel, E.T., Luderitz, O., and Westphal, O., Eur. J. Biochem. 9:143, 1971.

50. Loos, M., Bitter-Suermann, D., and Dierich, M., J. Immunol. 112:935, 1974.

51. Morrison, D.C., and Kline, L.F., J. Immunol. 118:367, 1977.

52. Verhoef ,J., Peterson, P.K., Kim, Y., Sabath, L.D., and Quie, P.G., Immunol. 33:191, 1977.

53. Bjornson, A.B., Altemeier, W.A., and Bjornson, H.S., Infect. and Immun. 14:843, 1976.

54. Bjornson, A.B., and Michael, J.G., J. Inf. Dis. 128:s182, 1973.

55. Bjornson, A.B., and Michael, J.G., J. Inf. Dis. 130:s119, 1974.

56. Winkelstein, J.A., Shin, H.S., and Wood, W.B.,Jr., J. Immunol. 108:1681, 1972.

57. Williams, R.C., Jr. and Quie, P.G., J. Immunol. 106:51, 1971.

58. Kierszenbaum, F., and Weinman, D., Immunol. 32:245, 1977.

59. Platt-Mills, T.A.E., and Ishizaka, K., J. Immunol. 113:348, 1974.

60. Joseph, B.S., Cooper, N.A., and Oldstone, M.B.A., J. Exp. Med. 141:761, 1975.

61. Kasper, D.L., J. Inf. Dis. 133:79, 1976.

62. Wilson, W.A., Hughes, G.R.V., and Lachmann, P.J., Brit. Med. J. 1:367, 1976.

63. Martin, A., Lachmann, P.J., Halbwachs, L., and Hobart, M.J., Immunochem. 13:317, 1976.

64. Tamura, N., and Nelson, R.A., J. Immunol. 99:582, 1967.

65. Lachmann, P.J., and Muller-Eberhard, H.J., J. Immunol. 100:691, 1968.

66. Ruddy, S., and Austen, K.F., J. Immunol. 107:742, 1971.

67. Fearon, D.T., and Austen, K.F., J. Exp. Med. 146:22, 1977.

68. Nelson, R.A., Jensen, J., Gigli, I., and Tamura, N., Immunochem. 3:111, 1966.

69. Hirsh, J.G., and Strauss, B., J. Immunol. 92:145, 1964.

70. Root, R.K., Ellman, L., and Frank, M.M., J. Immunol. 109:477, 1972.

71. Forsgren, A., and Quie, P.G., Infect. Immun. 10:402, 1974.

72. May, J.E., and Frank, M.M., Proc. Nat. Acad. Sci. 70:649, 1973.

73. Hill, A.W., Shears, A.L., and Hibbitt, K.G., Immunol. 34:131, 1978.

74. Mayer, M.M., Scientific Am. 299:54, 1973.

75. Guckian, J.C., Christenson, W.D., and Fine, D.P., Infect. Immun. 19:822, 1978.

76. Giebink, G.S., Verhoef, J., Peterson, P.K., and Quie, P.G., Infec. Immun. 18:291, 1977.

77. Peterson, P.K., Wilkenson, B.J., Kim, Y., Schemeling, D., and Quie, P.G., Infect. Immun. 19:943, 1978.

78. Mantovani, B., J. Immunol. 115:15, 1975.

79. Ehlenberger, A.G., and Nussenzweig, V., J. Exp. Med. 145:357, 1977.

80. Goldstein, I.M., Kaplan, H.B., Radin, A., and Frosch, M., J. Immunol. 117:1282, 1976.

81. Westphal, O., and Jann, K., in Methods in Carbohydrate Chemistry, Vol. 5, Edited by R. L. Whistler, Academic Press, New York, p. 83.

82. Leive, L., Shovlin, V.K., and Mergenhagen, S.E., J. Biol. Chem. 243:6384, 1968.

83. Westphal, O., Gmeiner, J. Luderitz, O., Tanaka, A., and Eichenberger, E., Colloq. Int. Cent. Natl. Reich. Sci. 174:69, 1969.

84. Borsos, T., and Rapp, H., J. Immunol. 99:263, 1967.

85. Pillemer, L., Schoenberg, M.D., Blum, L., and Wurz, L., Science 122:545, 1955.

86. Marcus, R.L., Shin, H.S., and Mayer, M.M., Proc. Nat. Acad. Sci. 68:1351, 1971.

87. Gotze, O., and Muller-Eberhard, H.J., J. Exp. Med. 134:90s, 1971.

88. Dierich, M.P., Bitter-Suermann, D., Konig, W., Hadding, U., Galanos, C., and Rietschel, E.T., Immunol. 24:721, 1973.

89. Fine, D.P., Marney, S.R., Colley, D.G., Sergent, J.S., and Des Pres, R.M., J. Immunol. 109:807, 1972.

90. Synderman, R., and Pike, M.C., Infect. Immun. 11:273, 1975.

91. Chang, H., Rodriguez, V., Narboni, G., Bodey, G.P., Luna, M.A., and Freierich, E.J., Medicine 55:259, 1976.

92. Rose, H.D., and Varkey, B., Medicine 54:499, 1975.

93. Young, R.C., Bennett, J.E., Geelhoed, G.W., and Levine, A.S., Ann. Intern. Med. 80:605, 1974.

94. Altemeier, W.A., Hummel, R.P., Hill, E.O., and Lewis, S., Ann. Surg. 178:436, 1973.

95. MacMillan, B.G., Law, E.J., and Holder, I.A., Arch. Surg. 104:509, 1972.

96. Gauto, A., Law, E.J., Holder, I.A., MacMillan, B.G., Am. J. Surg. 133:174, 1977.

97. Rippon, J.W., in Medical Mycology: the Pathogenic Fungi and the Pathogenic Actinomycetes, W.B. Saunders Co., Philadelphia, P. 191.

98. Hurley, D.L., and Fauci, A.S., J. Inf. Dis. 131:516, 1975.

99. Louria, D.B., Stiff, D.P., and Bennett, B., Medicine 41:307, 1962.

100. Medoff, G., Dismukes, W.E., Meade, R.H., III, and Moses, J.M., Arch. Intern. Med. 130:241, 1972.

101. Lehrer, R.I., Infect. Immun. 2:42, 1970.

102. Lehrer, R.I., and Cline, M.J., J. Clin. Invest. 48:1478, 1969.

103. Lehrer, R.I., and Cline, M.J., Cancer 27:1211, 1972.

104. Oh, M.K., Rodey, G.E., Good, R.A., Chilgren, R.A. and Quie, P.G., J. Pediatrics 75:300, 1969.

105. Quie, P.G., and Chilgren, R.A., Hematol. 8:227, 1971.

106. Bodey, G.P., J. Chronic Dis. 19:667, 1966.

107. Pearsall, N.N., Adams, B.L., and Bunni, R., J. Immunol. 120:1176, 1978.

108. Solomkin, J.S., Mills, E.L., Giebink, G.S., Nelson, R.D., Simmons, R.L., and Quie, P.G., J. Inf. Dis. 137:30, 1978.

109. Diamond, R.D. and Allison, A.C., Infect. Immun. 14:716, 1976.

110. Diamond, R.D., Krzesicki, R., and Wellington, J., J. Clin. Invest. 61:349, 1978.

111. Diamond, R.D., and Krzesicki, R., J. Clin. Invest. 61:360, 1978.

112. Bjornson, A.B., and Bjornson, H.S., J. Inf. Dis., in press.

113. Flodin, P., and Killander, J., Biochem. Biophys. Acta. 63:403, 1962.

114. Sober, H.A., Gutter, F.J., Wycoff, M.M., and Peterson, E.A., J. Amer. Chem. Soc. 78:756, 1956.

115. Davis, B.J., Ann. N.Y. Acad. Sci. 121:404, 1965.

116. Fearon, D.T., and Austen, K.F., J. Exp. Med. 140:426, 1974.

117. Casciato, D.A., Rosenblatt, J.E., Goldberg, L.S., and Bluestone, R., Infect. and Immun. 11:337, 1975.

118. Babb, J.L., and Cummins, C.S., Infect. and Immun. 19:1088, 1978.

DISTRIBUTION LIST

4 copies	HQDA (SGRD-AJ) Fort Detrick Frederick, MD. 21701
12 copies	Defense Documentation Center (DDC) ATTN: DDC-TCA Cameron Station Alexandria, Virginia 22314
1 copy	Dean School of Medicine Uniformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, Maryland 20014
1 copy	Superintendent Academy of Health Sciences, US Army ATTN: AHS-COM Fort Sam Houston, Texas 78234